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Volume 1
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The unsaturated fatty acids in menhaden body oil: the C_{18}, C_{20}, and C_{22} series*

W. Stoffel and E. H. Ahrens, Jr.
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[Received for publication August 8, 1959]

SUMMARY

Complete structural characterization of the C_{18}, C_{20}, and C_{22} unsaturated acids of menhaden body oil is described. The present report brings to 23 the number of chemically defined unsaturated acids in this oil, accounting for 95 per cent of the total weight of unsaturated acids. Of these 23 acids, 7 have not previously been reported. Pure acids (or mixtures of positional isomers) were isolated by fractional crystallization, fractional distillation, countercurrent distribution, rubber column chromatography, and preparative gas-liquid chromatography. Acids which were homogeneous in respect to chain length and number of double bonds were degraded by oxidative and reductive ozonolysis; fragments were identified by gas-liquid chromatography of the carboxylic acids and as addition-products of the aldehydes. Our studies of this complex oil, which is composed of more than 44 different acids, provide convincing evidence of the great usefulness of gas-liquid chromatography as a preparative and analytical procedure. Chemical characterization studies are now feasible on a microscale.

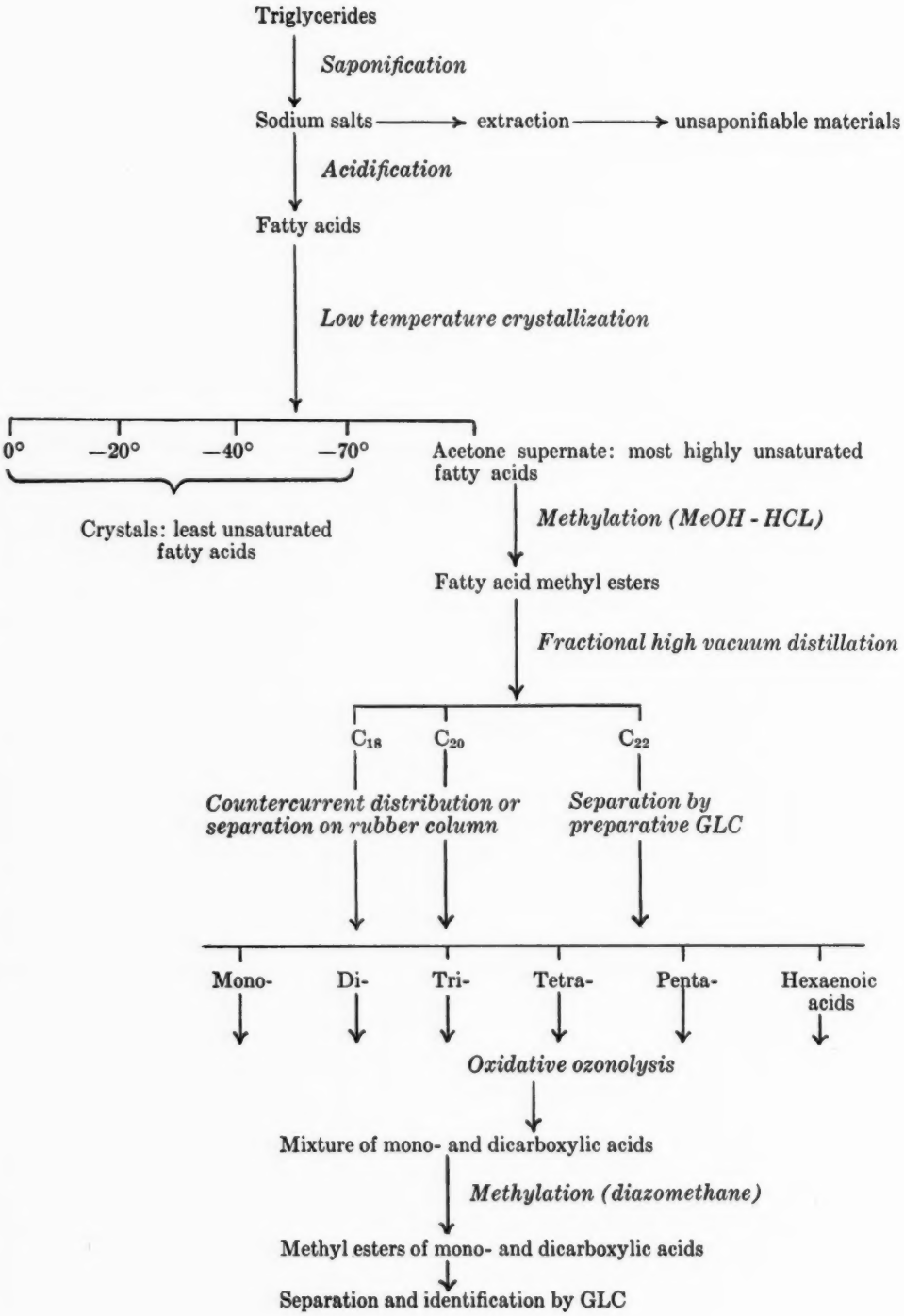
The present report marks the completion of a study of the fatty acid composition of menhaden body oil. Originally undertaken to complement an investigation of the nutritional effects of this marine oil in man (1), this study was aimed at the isolation and complete definition of the chemical structure of its unsaturated fatty acids. A previous report (2) described the C_{16} acids, and the present report the C_{18}, C_{20}, and C_{22} acids. We can account now for more than 95 per cent of all the unsaturated acids in this oil, based on our total quantitative analysis (1) by gas-liquid chromatography (GLC). Only the C_{14} monoene, traces of odd-numbered acids, and traces of three C_{22} acids remain uncharacterized. In the course of this work it was advantageous to determine the GLC characteristics of the acids isolated in pure form; their retention characteristics and stability on polar and nonpolar GLC columns are described elsewhere (3, 4).

Our isolation and identification procedures have been improved and adapted more to a microscale since the previous report (2). Figure 1 shows the present flow sheet. The starting material was especially prepared from fresh menhaden bodies and was not contaminated with head, liver, or visceral fats; a complete description of this preparation is given elsewhere, as well as a variety of chemical characteristics of the oil (1). Low temperature crystallization of the free fatty acids yielded the most highly unsaturated acids, which were then methylated and separated into chain-length groups (C_{18}, C_{20}, and C_{22}) by repeated fractional distillation (4). Only those fractions which were homogeneous in respect to chain length (verified by GLC after complete hydrogenation of a small sample of each) were used for further steps. The various unsaturated esters of each chain length were then separated into groups varying in number of double bonds (mono-, dienes, etc.), using a rubber column for the methyl esters (6) or countercurrent distribution (CCD) (7) of the free fatty acids. The homogeneity of each of these fractions in respect to number of double bonds was checked in three ways: (*a*) superimposition of theoretical and experimental CCD curves; (*b*) ultraviolet spectroscopy after isomerization by alkali (8); and (*c*) GLC on a polar stationary phase.

Having separated the original mixture of most highly unsaturated acids into chain-length groups and each of these into subfractions which were homogeneous in respect to number of double bonds, the final step consisted of identifying the double-bond structure of the acids in each of the subfractions. This was accomplished by oxidative ozonolysis with identification of all fragments by GLC. The fragment from the car-

* Supported in part by Grant H-2539 of the United States Public Health Service.

FIGURE 1. FLOW SHEET



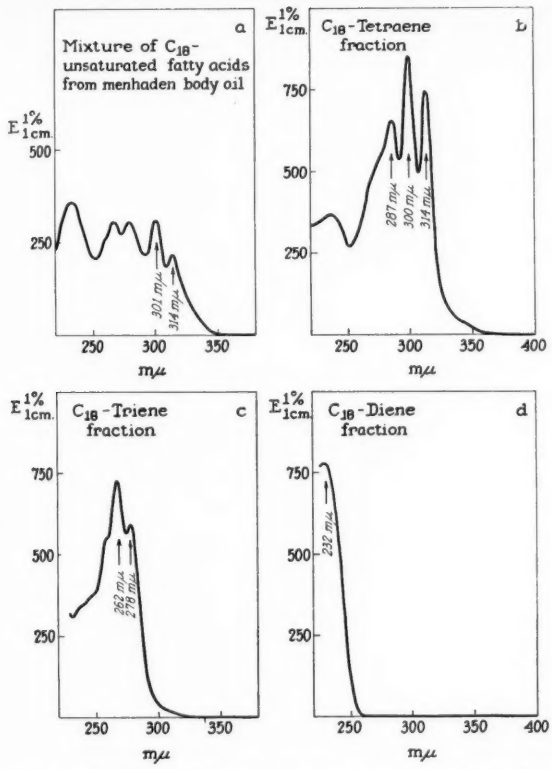


FIG. 2. C_{18} Acids—ultraviolet spectra after isomerization with alkali (8): (*a*) the C_{18} acid mixture; (*b*) the C_{18} tetraene (CCD peak D); (*c*) the C_{18} trienes (CCD peak C); (*d*) the C_{18} dienes (CCD peak B). Positions of peaks are italicized.

boxyl group to the nearest double bond ("carboxyl fragment") and the fragments between double bonds ("intermediate fragments") were identified as dicarboxylic acids, while the "terminal fragment" from the terminal methyl group to the nearest double bond was identified as a monocarboxylic acid. In a few critical cases, terminal fragments were identified and quantified as the 2,4-dinitrophenylhydrazones of the aldehydes produced by reductive ozonolysis (9). On the basis of these pieces of evidence the double-bond structures of the acids in each of the subfractions were assigned and their relative proportions calculated.

In the case of the C_{22} acids, it proved difficult to separate the mixture into cleanly resolved peaks by CCD. However, it was possible to use GLC as a preparative method because of the wide separation between pentaenes and hexaenes. By micro-ozonolysis of these fractions[1] and GLC of the mono- and dicarboxylic acids formed, their double-bond structures were defined.

[1] W. Stoffel, F. Chu, and E. H. Ahrens, Jr. Manuscript in preparation.

Table 1 lists the fragments determined by oxidative and reductive ozonolysis of the C_{18}, C_{20}, and C_{22} unsaturated fatty acids in menhaden body oil and the proposed structures of the fatty acids in these groups. The C_{16} acids previously described (2) are included for sake of completeness. Many of the acids in Table 1 have been characterized previously by other workers, having been isolated from a wide variety of biological sources; references to this literature are included. However, seven of the acids listed have not been described before.

EXPERIMENTAL

Separation by Chain Length. Fractional high vacuum distillation (column length 750 mm., 10^{-4} mm. Hg pressure at the distillation head) (5) was carried out on about 50 g. of mixed esters. Small samples of distillate were hydrogenated completely with Adams catalyst (PtO_2) and the homogeneity of chain length checked by GLC. The fractions shown to be exclusively C_{18} and C_{20}, respectively, were used for further investigation. The esters were colorless oils. The C_{18} fraction had an iodine value (I.V.) of 201, the C_{20} fraction 290. Ultraviolet spectroscopy showed absence of conjugated acids, and infrared spectroscopy absence of *trans* acids. After isomerization with alkali, tetraenoic C_{18} and pentaenoic C_{20} acids were shown to be the most unsaturated components in their respective fractions (Figs. 2 and 3).

Separation by Degree of Unsaturation. Mixed C_{18} esters (5.20 g.) and mixed C_{20} esters (5.15 g.) were saponified, and the free fatty acids were separated by CCD (7) into four groups of acids in each case, differing from each other only in the number of double bonds. A 200-tube all-glass fully automatic machine with 10 ml. lower phase volume was used with a solvent system of n-heptane : methanol : acetonitrile : acetic acid (3 : 1 : 1 : 1). After 680 transfers in the C_{18} distribution, alkali isomerization of aliquots of each peak gave evidence that *D* in Figure 4 was the tetraene, *C* the triene, and *B* the diene group. That peak *A* (tubes 220 to 250) contained the monoene was established by the I.V. of 88; in addition, its GLC retention time on a polar stationary phase (ethylene glycol adipate polyester, EGA) was identical to that of methyl oleate.

In the case of the C_{20} distribution, the dienes and trienes (Fig. 5, peaks *C* and *B*) were well separated in 400 transfers and were removed; after 660 transfers of the remaining material the tetraenes (A_2) and pentaenes (A_1) were separated. The tetraene peak was removed and the distribution of the pentaene continued

TABLE 1. STRUCTURES OF UNSATURATED FATTY ACIDS IN MENHADEN BODY OIL. TABLE DEMONSTRATES EVIDENCE ON WHICH ARE BASED THE STRUCTURES OF 23 ACIDS, COMPRISING 95% OF THE TOTAL WEIGHT OF UNSATURATED ACIDS IN THIS OIL.

Chain length	Fatty acids fraction	I.V. (22)	Degradation products after oxidative ozonolysis		Aldehydes obtained after reductive ozonolysis	Position of double bonds (numbered from carboxyl carbon)	Approximate relative amounts of isomers	Percentage in original menhaden body oil *	Characterized previously
			Monocarboxylic acids	Dicarboxylic acids					
C_{16} (I.V. 180–185)	tetraenoic	405		adipic malonic succinic		6,9,12,15 4,7,10,13	20 1	1.9 0.1	South African pilchard oil (10) herring oil †
	trienoic	302		pimelic adipic malonic	butyraldehyde propionaldehyde acetaldehyde	6,9,12 7,10,13	5 1	1.1 0.2	rape seed oil (11) ‡
	dienoic	202	butyric heptanoic	azelaic adipic malonic	butyraldehyde heptylic aldehyde	6,9 9,12	1 4	0.4 1.6	‡ ‡
	monoenoic	103	heptanoic caprylic	azelaic suberic		8 9	1 6	1.4 8.4	‡ numerous sources (12)
C_{18} (I.V. 201)	tetraenoic	354	propionic	malonic adipic	propionaldehyde	6,9,12,15		3.2	herring oil (13)
	trienoic	266	propionic caproic	malonic adipic azelaic	propionaldehyde capronaldehyde	9,12,15 6,9,12	4 1	1.0 0.3	linseed oil (12), herring oil (9) ‡
	dienoic	176	caproic pelargonic	malonic azelaic adipic		9,12 6,9	4 1	2.2 0.5	herring oil (9) ‡
	monoenoic	88	pelargonic	azelaic		9		14.5	numerous sources (12)
C_{20} (I.V. 290)	pentaenoic	405	propionic	malonic glutaric	propionaldehyde	5,8,11,14,17		12.5	cod liver oil (14) ox liver phosphatides (15)
	tetraenoic	326	propionic caproic	malonic glutaric suberic	propionaldehyde capronaldehyde	5,8,11,14 8,11,14,17	2 3	0.2 0.4	ox liver phosphatides (15) ‡
	trienoic	245	caproic pelargonic	malonic glutaric suberic		5,8,11 8,11,14	1 1	1.0 1.0	ox liver phosphatides (15) ox liver phosphatides (15), rat tissues (16
	dienoic	163	caproic pelargonic	malonic suberic undecane-dicarb.		8,11 11,14	1 1	0.6 0.6	ox liver phosphatides (15)
C_{22}	hexaenoic		propionic § oxalic	malonic succinic		4,7,10,13,16,19		8.9	herring oil (19), pilchard oil (17), cod liver oil (18), ox liver phosphatides (19
	pentaenoic		propionic	malonic pimelic		7,10,13,16,19		2.0	cod liver oil (18), pilchard oil (20), herring oil (9), ox brain phosphatides (21) ox liver phosphatides (19)

* Calculations based on the total analysis given elsewhere (1) and on the ratios in the column to the left. For example, in Reference (1), C_{16} tetraenoic acid is reported to constitute 2.0% of total fatty acids, and since this fraction is stated in the present study to be made up of two isomers in proportions of 20/1 (approximate), the per cent of each acid in the whole oil is 1.9 and 0.1% respectively.

† E. Klenk, personal communication. ‡ Acids not previously characterized. § Artifact, see text.

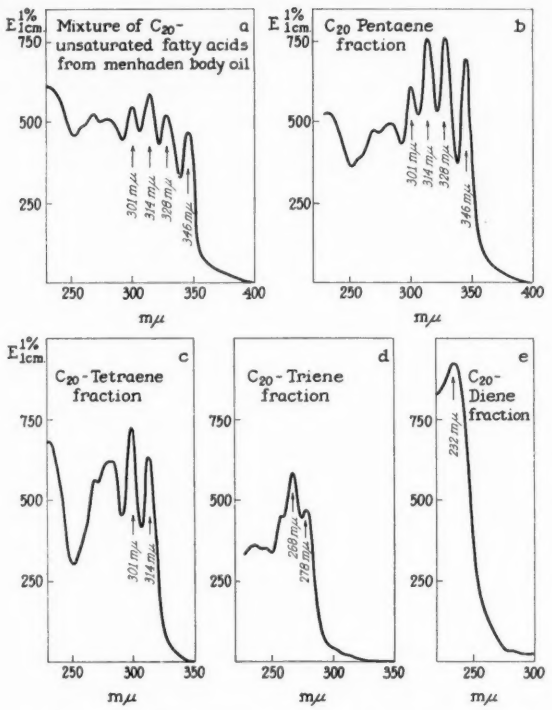


FIG. 3. C_{20} Acids—ultraviolet spectra after isomerization with alkali (8): (*a*) the C_{20} acid mixture; (*b*) the C_{20} pentaene (CCD, tubes 380 to 430); (*c*) the C_{20} tetraenes (CCD, tubes 460 to 500); (*d*) the C_{20} trienes (CCD peak B); (*e*) the C_{20} dienes (CCD peak C). Positions of peaks are italicized.

for 975 transfers without demonstrating any further peaks. The partition coefficient (K) of the pentaene remained constant between 700 and 975 transfers, and the theoretical and experimental CCD curves were superimposable.

The ultraviolet absorption spectra after isomerization with alkali (8) of the total mixture and of the different groups isolated by CCD are given in Figure 2 for the C_{18} series, in Figure 3 for the C_{20} series, and in Figure 6 for the C_{22} series. It is evident that there was no contamination of any fraction by its more highly unsaturated homologue.

The methyl esters of the C_{18} and C_{20} chain-length groups also were separated into groups, homogeneous in respect to numbers of double bonds, by chromatography on columns (1 × 150 cm.) of finely ground natural rubber (6), eluting with 10 and 12 per cent water in acetone, respectively. Fifty-milligram samples of the mixed methyl esters were chromatographed; the chromatograms were monitored by an automatic recording differential refractometer; fractions were collected automatically. The separations obtained were identical to those produced by CCD. Sufficient material was present in the various peaks for definitive degradation studies by micro-ozonolysis.[2]

The residue of the fractional distillation of C_{20} acids was subjected to preparative GLC on an Apiezon-M column at 197°C, utilizing an ionization chamber detector (4). Five samples (about 2 mg. each) were chromatographed. Despite overloading of the column, an excellent separation of C_{22} penta- and hexaenoic acid methyl esters was permitted by the very different retention times of these esters (3, 4). The contaminating C_{20} acids and C_{22} acids present only in trace amounts were eluted well ahead of the two major C_{22} acids. These major acids were separately collected; their chain length was checked by complete hydrogenation (4), followed by GLC of the n-saturated products, while the number of double bonds in each fraction was verified by ultraviolet spectroscopy after

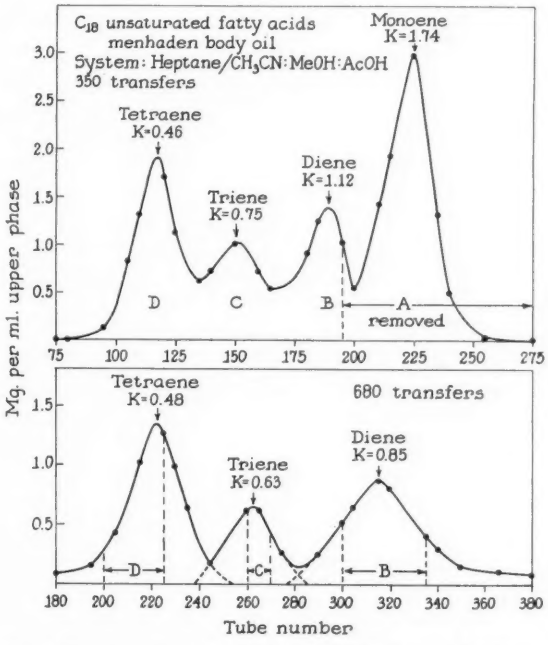


FIG. 4. Countercurrent distribution (CCD) pattern of 5.20 g. of C_{18} unsaturated fatty acids from menhaden body oil. Fractions *B-D* were harvested as shown, fraction *A* from tubes 220 to 250.

isomerization with alkali. Sufficient materials remained for oxidative ozonolysis.

Procedure of Oxidative Ozonolysis and Identification of Fragments. When 20 to 25 mg. of acids were available for ozonolysis, the procedure previously described (2) was used; for 0.5 to 5 mg. samples a micro-

[2] See footnote 1, p. 141.

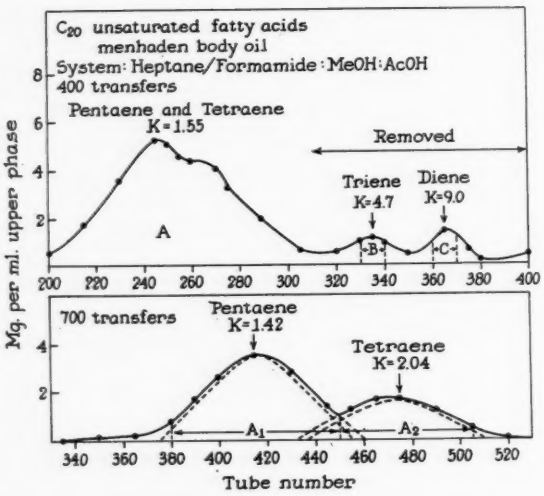


FIG. 5. CCD pattern of 5.18 g. of C_{20} unsaturated fatty acids from menhaden body oil. Fractions *B* and *C* were harvested as shown, fraction A_1 from tubes 380 to 430, fraction A_2 from tubes 460 to 500.

technic was applied.[3] The mixed mono- and dicarboxylic acids produced were dissolved in ethyl ether and methylated with diazomethane in ether in the usual manner (23). After evaporation of solvent, the residue was analyzed by GLC.

GLC was carried out (4) with an ionization chamber detector, argon as moving phase and EGA as stationary phase. Long-chain mono- and C_2-C_9 dicarboxylic acid methyl esters were best resolved at 173°C, the shorter di- and all monocarboxylic acids at 78°C. For identification of fragments produced from polyethenoic acids it was necessary to make analyses at both temperatures. In all cases retention times matched those of standard samples of C_2-C_9 methyl esters of pure mono- and dicarboxylic acids.

It was recognized that the recoveries of C_2-C_5 monocarboxylic acid methyl esters were less than theoretical, due to losses incurred when solvents were evaporated. When it was important to establish molar ratios of the various fragments produced by ozonolysis, the terminal fragments were recovered quantitatively after reduction to the aldehydes and formation of the 2,4-dinitrophenylhydrazones (9).

RESULTS

All fractions were obtained as colorless oils.

C_{18}-tetraenoic acid fraction (fraction *D*, Fig. 4): n_D^{22} 1.4861, I.V. 354 (22) (calculated for $C_{18}H_{28}O_2$, 362); $E^{1\%}_{1\,cm.}$ = 860, ϵ_{max} 23700 at λ_{max} 300 mμ. Oxidative ozonolysis yielded propionic, malonic, and adipic acids. On reductive ozonolysis propionaldehyde was the only aldehyde derived as a terminal fragment. The results indicated a single C_{18}-tetraene with double bonds in the 6,9,12,15 positions (numbered from the carboxyl carbon).

C_{18}-trienoic acid fraction (fraction *C*, Fig. 4): n_D^{22} 1.4665, I.V. 266 (calculated for $C_{18}H_{30}O_2$, 272); $E^{1\%}_{1\,cm.}$ = 725, ϵ_{max} 19900 at λ_{max} 268 mμ. Oxidative ozonolysis yielded propionic, caproic, malonic, adipic, and azelaic acids. On the basis of the ratio of adipic to azelaic acid, the two isomers (9,12,15- and 6,9,12-octadecetrienoic acids) were present in proportions of about 4 : 1. Reductive ozonolysis yielded propionaldehyde and capronaldehyde, also in a ratio of 4 : 1.

C_{18}-dienoic acid fraction (fraction *B*, Fig. 4): n_D^{22} 1.4600, I.V. 176 (calculated for $C_{18}H_{32}O_2$, 180); $E^{1\%}_{1\,cm.}$ = 775, ϵ_{max} 21400 at λ_{max} 232 mμ. Oxidative ozonolysis yielded caproic, pelargonic, malonic, adipic, and azelaic acids, indicating the presence of octadeca-6,9- and octadeca-9,12-dienoic acids in proportions of 1 : 4.

C_{18}-monoenoic acid fraction (fraction *A*, tubes 220 to 250, Fig. 4): n 1.4525, I.V. 88 (calculated for $C_{18}H_{34}O_2$, 89). Oxidative ozonolysis yielded only pelargonic and azelaic acids, indicating the structure octadeca-9-enoic (oleic acid) as the sole C_{18}-monoene.

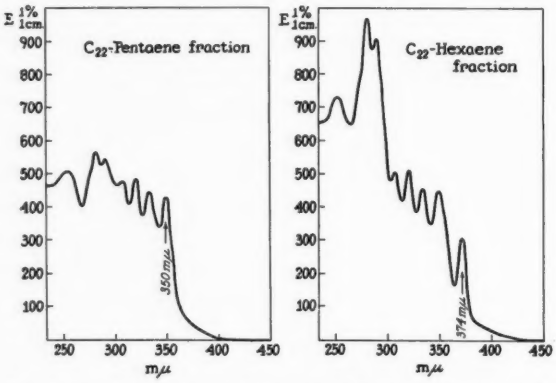


FIG. 6. C_{22} Acids—ultraviolet spectra after isomerization with alkali (8). Left, C_{22} pentaene; right, C_{22} hexaene (both isolated by preparative gas-liquid chromatography). Peak maxima are shown with arrows.

C_{20}-pentaenoic acid fraction (fraction A_1, tubes 380 to 430, Fig. 5): n_D^{22} 1.4890, I.V. 405 (calculated for $C_{20}H_{30}O_2$, 416); $E^{1\%}_{1\,cm.}$ = 695, ϵ_{max} 21400 at λ_{max} 346 mμ. Glutaric, malonic, and propionic acids were obtained by oxidative ozonolysis, and propionaldehyde was obtained by reductive ozonolysis. The results indicated a single pentaene with the 5,8,11,14,17 double-bond structure.

[3] See footnote 1, p. 141.

C_{20}-tetraenoic acid fraction (fraction A_2, tubes 460 to 500, Fig. 5): n_D^{22} 1.4880, I.V. 326 (calculated for $C_{20}H_{32}O_2$, 334); $E^{1\%}_{1\,cm.}$ = 725, ϵ_{max} 22000 at λ_{max} 301 mμ. Oxidative ozonolysis yielded propionic, caproic, malonic, glutaric, and suberic acids. On reductive ozonolysis propionaldehyde and capronaldehyde were obtained. The results indicated 5,8,11,14- (arachidonic) and 8,11,14,17-acids in proportions of about 2 : 3.

C_{20}-trienoic acid fraction (fraction *B*, Fig. 5): n_D^{22} 1.4681, I.V. 245 (calculated for $C_{20}H_{34}O_2$, 248); $E^{1\%}_{1\,cm.}$ = 600, ϵ_{max} 18400 at λ_{max} 268 mμ. Oxidative ozonolysis yielded pelargonic, caproic, malonic, glutaric, and suberic acids. The two dicarboxylic acids were present in about equal amounts. These results indicated the presence of eicosa-8,11,14-trienoic and eicosa-5,8,11-trienoic acids in proportions of 1 : 1.

C_{22}-hexaenoic acid fraction (preparative GLC): $E^{1\%}_{1\,cm.}$ = 300, ϵ_{max} 10000 at λ_{max} 374 mμ. Oxidative ozonolysis yielded oxalic, propionic, malonic, and succinic acids. Oxalic acid was present in only trace amounts and is considered an artifact: the mixed acids soluble in acetone at —70°C contained no preformed conjugation by ultraviolet spectroscopy, but after fractional distillation and GLC, the C_{22}-hexaene fraction showed 8 to 10 per cent conjugation. Discounting the oxalic acid formed by oxidative ozonolysis of the conjugated double bonds, the results indicated the presence of the 4,7,10,13,16,19-acid as the sole component of this fraction.

C_{22}-pentaenoic acid fraction (preparative GLC): $E^{1\%}_{1\,cm.}$ = 510; ϵ_{max} 17600 at λ_{max} 329 mμ. Oxidative ozonolysis yielded malonic, pimelic, and propionic acids, thus indicating that docosa-7,10,13,16,19-pentaenoic acid was the sole component of this fraction.

DISCUSSION

It is striking that for every polyethenoid acid in this sample of menhaden body oil a double-bond arrangement of the divinyl methane or methylene-interrupted type (=CHCH$_2$CH=) was found. Moreover, we found no evidence of *trans* acids; conjugated double bonds were detected only in the C_{22} hexaenes in small amounts, under circumstances which suggested artifactual changes. In their studies of other marine oils and fats from mammalian sources, Klenk and Bongard (24) have commented on the consistency of divinyl methane rhythm of double bonds in naturally occurring nonconjugated polyenes. Previously, work by Japanese investigators (25, 26) had suggested that some marine polyenes show both methylene- and ethylene-interruption, but this conclusion is currently ascribed to certain laboratory artifacts (27). In the present study, total absence of ethylene-interrupted double bonds in menhaden polyethenoid acids is demonstrated in three ways: (*a*) typical ultraviolet patterns were seen after isomerization by alkali, yet de Surville *et al.* (28) have shown that ethylene-interrupted double bonds prevent the occurrence of isomerization under the conditions used; (*b*) upon oxidative ozonolysis, succinic acid would be produced as an intermediate fragment from a divinyl ethane grouping as well as malonic acid from a divinyl methane grouping, yet Table 1 indicates that succinic acid was found only twice (C_{16} tetraene and C_{22} hexaene) and there as carboxyl fragments; and (*c*) the quantitative results obtained after reductive ozonolysis substantiated in every case the qualitative information obtained by oxidative ozonolysis.

In fatty acids of unknown structure it is possible to define double-bond structure if only three pieces of information are assembled (assuming that conjugated double bonds have been ruled out by ultraviolet spectroscopy): (*a*) the number of double bonds by ultraviolet spectroscopy after isomerization with alkali, (*b*) the chain length of the acid shown by GLC after complete hydrogenation, and (*c*) the identity of the carboxyl fragment after oxidative ozonolysis. Micromethods have been developed which permit the investigator to obtain this information on 3 mg. or less of an unknown acid;[4] the value of GLC in microanalyses of this type cannot be overemphasized. The validity of this shortened procedure for determination of double-bond structure depends, of course, on the assumption that double bonds occur exclusively in divinyl methane rhythm.

It is intriguing to note certain structural similarities among the acids in Table 1. If the double bonds are numbered from the terminal rather than the carboxyl carbon, as suggested by Thomasson (29), it is seen that most of the acids are of three types: oleic (Δ 9,10-terminal), linoleic (Δ 6,9-terminal), and linolenic (Δ 3,6,9-terminal). The most conspicuous exceptions occur among the C_{16} acids, where the unusual positioning of the double bonds suggests the possibility that these acids may have been formed from C_{18} acids by loss of two terminal carbons, or by dehydrogenation of palmitoleic acid as suggested by Fulco and Mead (30).

The total unsaturated acids in menhaden body oil comprise 70 per cent of the whole, yet these acids are by no means evenly distributed in regard to chain length or to degree of unsaturation. For example, the C_{16} unsaturated acids make up 15 per cent; C_{18}, 22

[4] See footnote 1, p. 141.

per cent; C_{20}, 16 per cent; and C_{22}, 11 per cent of the total acids. Moreover, among these chain length groups it is interesting to note that the monoenes predominate in the C_{16} and C_{18} groups, while in the C_{20} and C_{22} groups the penta- and hexaenes are present in highest concentration.

Lacking information on the fatty acid structure of the plankton upon which menhaden feed, it is impossible to know to what extent the fatty acids of menhaden body oil reflect the synthetic or rearrangement processes of the fish or of the algae. Yet it is tempting to speculate that the C_{16} and C_{18} monoenes, both Δ-9 acids, are the progenitors of the more highly unsaturated acids, and that the final pattern of distribution of acids represents the competition between chain elongation by addition of acetate units and progressive desaturation toward both ends of the fatty acid chain, starting from the Δ 9 double bond. If C_{18} monoene is elongated by addition of acetate, the Δ 9 bond in the C_{18} acid becomes Δ 11 in the C_{20} series, and Δ 13 in the C_{22} series. Thereafter, most of the acids shown in Table 1 could have been formed by progressive stepwise dehydrogenation (always in divinyl methane rhythm) on either side of the pivotal double bond.

The very small amounts of "essential fatty acids" in this oil have been noted in a previous publication (1); the total amount of linoleic-type acids was 2.6 per cent, a figure corroborated by the bioassay value of 4 units.

REFERENCES

1. Ahrens, E. H., Jr., W. Insull, Jr., J. Hirsch, W. Stoffel, M. L. Peterson, J. W. Farquhar, T. Miller, and H. J. Thomasson. *Lancet* **1**: 115, 1959.
2. Stoffel, W., and E. H. Ahrens, Jr. *J. Am. Chem. Soc.* **80**: 6604, 1959.
3. Stoffel, W., W. Insull, Jr. and E. H. Ahrens, Jr. *Proc. Soc. Exptl. Biol. Med.* **99**: 238, 1958.
4. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. *Nutrition Revs.* **17**: 8, 1959 (Suppl.).
5. Klenk, E. In *Handbuch der physiologisch- und pathologisch-chemischen Analyse,* edited by F. Hoppe-Seyler and H. Thierfelder, Berlin, Springer-Verlag, 1955, vol. 3, p. 447.
6. Hirsch, J. *Federation Proc.* **18**: 246, 1959.
7. Ahrens, E. H., Jr., and L. C. Craig. *J. Biol. Chem.* **195**: 299, 1952.
8. Holman, R. T. *Methods of Biochem. Anal.* **4**: 99, 1957.
9. Klenk, E., and H. Brockerhoff. *Z. physiol. Chem. Hoppe-Seyler's* **310**: 153, 1958.
10. Silk, M. H., and H. H. Hahn. *Biochem. J.* **57**: 582, 1954.
11. Shorland, F. B. *Nature* **156**: 269, 1945.
12. Hilditch, T. P. *The Chemical Constitution of Natural Fats,* 3d ed., New York, John Wiley & Sons, Inc., 1956, p. 483.
13. Klenk, E., and H. Brockerhoff. *Z. physiol. Chem. Hoppe-Seyler's* **307**: 272, 1957.
14. Klenk, E., and D. Eberhagen. *Z. physiol. Chem. Hoppe-Seyler's* **307**: 42, 1957.
15. Klenk, E., and W. Montag. *Ann. Chem. Liebigs* **604**: 4, 1957.
16. Mead, J. F., and W. H. Slaton, Jr. *J. Biol. Chem.* **219**: 705, 1956.
17. Whitcutt, J. M. *Biochem. J.* **67**: 60, 1957.
18. Klenk, E. *1st Internat. Conf. on Biochem. Problems of Lipids,* Brussels, 33 (1953).
19. Klenk, E., and H. J. Tornuschat. *Z. physiol. Chem. Hoppe-Seyler's* **308**: 165, 1957.
20. Whitcutt, J. M., and D. A. Sutton. *Biochem. J.* **63**: 469, 1956.
21. Klenk, E., and F. Lindlar. *Z. physiol. Chem. Hoppe-Seyler's* **299**: 74, 1955.
22. Rosenmund, K. W., and W. Kuhnhenn. *Z. Nahr. Genussm.* **46**: 154, 1923.
23. Vogel, A. I. *Textbook of Practical Organic Chemistry,* 3d ed., New York, Longmans, Green & Co., Inc., 1956.
24. Klenk, E., and W. Bongard. *Z. physiol. Chem. Hoppe-Seyler's* **291**: 104, 1952.
25. Matsuda, S. *J. Soc. Chem. Ind. Japan* **45**: 158, 1942 (Suppl. binding).
26. Tutiyâ, T. *J. Chem. Soc. Japan* **61**: 717, 867, 1188, 1940; **62**: 10, 552, 1941.
27. Hilditch, T. P. *The Chemical Constitution of Natural Fats,* 3d ed., New York, John Wiley & Sons, Inc., 1956, pp. 513, 539.
28. de Surville, B. M. A., D. E. A. Rivett and D. A. Sutton. *J. Chem. Soc.,* Part III: 3304 (1957).
29. Thomasson, H. J. *1st Internat. Conf. on Biochem. Problems of Lipids,* Brussels, 212 (1953).
30. Fulco, A. J., and J. F. Mead. *J. Biol. Chem.* **234**: 1411, 1959.

Volume 1
Number 2

The lipids of the myocardium, conducting bundle, and valves of beef heart*

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SUMMARY

The lipids of myocardium, conducting bundle, and valves of beef heart were investigated. The atrioventricular valves contained the highest content of total lipid, most of which was triglyceride. The lipids of myocardium and semilunar valves contained the highest amount of phosphatides and cholesterol. The plasmalogens were localized predominantly in the phosphatide fraction of the myocardium and conducting bundle. An analysis of the individual phosphatides showed that with respect to the total lipid phosphorus, the polyglycerolphosphatide was localized in the myocardium and conducting bundle, sphingomyelin occurred in highest amount in the conducting bundle, and lecithin occurred in highest amount in the valves.

Data on the lipids of heart tissues are given by Mallov and co-workers (1) and are summarized by Deuel (2). Little information is available on the amount of each specific lipid, in particular the phosphatides. In previous studies quantitative paper and column methods for phosphatide analysis were employed to study the individual phosphatides of pig heart muscle (3) and the cell fractions of pig heart (4).

In this paper the lipids of ventricular myocardium, conducting bundle, and valves of beef heart were investigated. The data show significant differences in the lipid distribution in the various heart tissues and emphasize the distinctive pattern of lipid localization in this organ.

METHODS

Two beef hearts obtained from animals immediately after slaughter were maintained on ice until ready for extraction. Portions of myocardium were removed from the apex of the ventricles. The larger proximal portions of the conducting bundle were carefully dissected from surrounding muscle and connective tissue. The semilunar (pulmonary and aortic) valves and the atrioventricular (tricupid and mitral) valves were removed and freed from muscle, extraneous connective tissue, and fat.

The myocardium, conducting bundle, and valves were minced and extracted with chloroform-methanol as described previously (4). Column and paper chromatography of the lipids were carried out by the method of Marinetti and co-workers (3, 4). Cholesterol analysis was performed on the nonphosphatide fraction obtained by column chromatography. The Liebermann-Burchard reaction was employed as described previously (5). Plasmalogens were detected by the Schiff test (3).

RESULTS

The data on the lipid content of beef heart tissues are summarized in Tables 1 to 3. The experiments were repeated three times with essentially the same results. The data in Table 1 give the gross analytical picture of the percentage distribution of the major lipid groups on the basis of dry weight tissue. The high lipid content of the atrioventricular valves is noteworthy, particularly in view of the relatively low lipid content of the semilunar valves.

A more detailed analysis of the lipids of the heart tissues is given in Table 2. The content of the lipid components is expressed on a relative basis, namely, relative to the total dry weight of lipid, rather than to the dry weight of tissue. These data clearly show that the lipids of myocardium and semilunar valves are rich in phosphatides, whereas the lipids of the atrioventricular valves are predominantly nonphosphatides, in particular triglycerides. The difference in the relative cholesterol content of the lipids of the heart valves is also apparent.

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The data in Table 2 demonstrate a qualitative difference in the plasmalogen[1] content of the heart tissues. The plasmalogens are present in the phosphatide fraction of all the tissues but they occur in higher amount in the myocardium and conducting bundle. The plasmalogen content of pig and beef heart has been published elsewhere (6, 7). A small amount of Schiff positive material was also found in the nonphosphatide fraction of myocardium and semilunar valves. In part, some of this lipid material is believed to represent free aldehydes that are liberated as a result of hydrolysis of the native plasmalogens either during the lipid extraction or during column fractionation.

TABLE 1. GROSS LIPID CONTENT OF BEEF HEART TISSUES

	Myocardium	Conducting Bundle	Valves *	
			A-V	S-V
Total dry weight tissue in mg.	1051.0	270.0	1088.0	590.0
Total lipid in mg.	128.0	31.0	248.0	22.0
Total lipid in % dry weight	12.1	11.5	22.8	3.7
Phosphatide † in % dry weight	6.6	2.7	1.2	1.8
Total cholesterol in % dry weight	0.8	0.45	0.38	0.65

* A-V represents atrioventricular valves; S-V represents semilunar valves.

† The phosphatides were determined by column fractionation (see Table 2). This fraction also includes cerebrosides.

The phosphorus content of the phosphatide fraction of the heart tissues was low and variable. If this fraction consisted only of the typical phosphatides, the per cent phosphorus should be between 3.6 to 4.0. Hence, it is clear that this fraction which is eluted by chloroform-methanol and methanol from silicic acid columns contains some polar nonphosphatides. Part of this material is accounted for by cerebrosides (3).

The analysis of the individual phosphatides is given in Table 3. The amount of each phosphatide is expressed as the per cent phosphorus which it comprises of the total lipid phosphorus. It can be seen that the polyglycerolphosphatide[2] occurs predominantly in the myocardium and in the conducting bundle. We have previously shown that this phosphatide is localized in mitochondria (4) and, moreover, in the rat occurs in highest amount in heart muscle (8).

The lipids of the conducting bundle were found to contain less phosphatidylethanolamine than the lipids of myocardium. In the case of the heart valves phosphatidylethanolamine and phosphatidylserine were not sufficiently resolved on paper chromatograms to analyze them separately. However, phosphatidylethanolamine occurred in higher amount in both cases.

The component having the chromatographic mobility and staining properties of inositol phosphatide was not completely separated from sphingomyelin in the case of myocardium. Hence, these two phosphatides were analyzed together. Sphingomyelin was, however, the major component.

Although precautions were taken during the extraction of the lipids to prevent their degradation, the myocardium lipids were found to contain an appreciable amount of lysolecithin. Since the nonphosphatide

TABLE 2. COLUMN CHROMATOGRAPHY OF BEEF HEART TISSUE LIPIDS *

	Myocardium	Conducting Bundle	Valves	
			A-V	S-V
I. Total lipid put on column in mg.	22.0	30.0	39.0	17.0
II. Nonphosphatides † in mg.	8.0	22.0	36.0	8.0
III. Phosphatides ‡ in mg.	12.0	7.0	2.0	8.0
Phosphatide content of I in %	54.5	23.3	5.1	47.2
Cholesterol content of I in %	6.6	3.9	1.7	17.4
Cholesterol content of II in %	18.0	5.3	1.8	37.0
Phosphorus content of III in %	2.6	1.3	1.4	1.0
Schiff test on II	++	neg.	neg.	+
Schiff test on III	++++++	+++	+	+

* Column fractionation was carried out on silicic acid (10 g.) as described previously (3).

† Represents the lipid fraction eluted with chloroform. This fraction has no phosphorus and contains mainly cholesterol, cholesterol esters, and triglycerides. These lipids were identified by paper chromatography (4).

‡ Represents the lipid fraction eluted with chloroform-methanol 1:1 and by methanol. This fraction also contains cerebrosides (3).

[1] The plasmalogen test with the Schiff reagent may not be specific for these particular phosphatides; rather it measures total available aldehyde.

[2] This lipid is believed to be the same as cardiolipin (4). In heart muscle and most other tissues it consists of two to four components having similar structure and properties.

fraction of myocardium (Table 2) contained Schiff positive material, it was concluded that the lysolecithin arose as a result of hydrolysis of the choline-plasmalogens. The relatively low content of lecithin in this sample supports this conclusion. Subsequent extractions of other beef hearts have shown that under carefully controlled conditions the amount of lysolecithin in myocardium is much smaller (less than 5 per cent of the total lipid phosphorus).

TABLE 3. PHOSPHATIDE COMPOSITION OF BEEF HEART TISSUES *

Phosphatide	Percentage of Total Lipid Phosphorus †			
	Myocardium	Conducting Bundle	Valves	
			A-V	S-V
Polyglycerolphosphatide	8	4	trace	trace
Phosphatidylethanolamine ‡	20	11	20	18
Phosphatidylserine	6	8		
Lecithin ‡	28	39	46	47
Sphingomyelin	24	26	23	20
Inositol phosphatide		5	7	8
Lysolecithin	13	trace	trace	trace
Others §	1	7	4	7

* Quantitative paper chromatography was carried out as described previously (3).

† The total lipid phosphorus is the sum of the phosphorus in all the phosphatide spots, including the origin material.

‡ These phosphatides contain the plasmalogens indicated in Table 2 as the Schiff positive material. The actual content of the plasmalogens of beef heart myocardium have been published elsewhere (7).

§ Trace amounts of other phosphatides were observed on the chromatograms. One of these is believed to be lysophosphatidylethanolamine. This fraction also includes 1 to 2 per cent material at the origin.

DISCUSSION

These experiments demonstrate the distinctive distribution of lipids in various tissues of beef heart. The lipid content of atrioventricular valves was high and consisted mainly of triglycerides. On the other hand, the lipids of semilunar valves consisted mainly of phosphatide and cholesterol. The response of these valves to pathogenic organisms may in part be attributed to this marked difference in lipid composition.

The plasmalogens were found in higher concentration in the myocardium and conducting bundle than in the valves. In addition, the polyglycerolphosphatides occurred nearly exclusively in the myocardium and conducting bundle. Since this lipid fraction is localized in mitochondria (4) and occurs in highest concentration in the heart (8), it may play a special role in heart metabolism.

The high content of sphingomyelin in the conducting bundle is noteworthy. In this respect this tissue appears to resemble nerve tissue, yet its morphology is similar to that of myocardium. It would be of interest if the conducting properties of the bundle of His were due to specific lipids.

REFERENCES

1. Mallov, S., J. M. McKibbin and J. S. Robb. *J. Biol. Chem.* **201**: 825, 1953.
2. Deuel, H. J. *The Lipids*, New York, Interscience Publishers, Inc., 1955, vol. 2.
3. Marinetti, G. V., J. Erbland and J. Kochen. *Federation Proc.* **16**: 837, 1957.
4. Marinetti, G. V., J. Erbland and E. Stotz. *J. Biol. Chem.* **233**: 562, 1958.
5. Marinetti, G. V., D. J. Scaramuzzino and E. Stotz. *J. Biol. Chem.* **224**: 819, 1957.
6. Marinetti, G. V., J. Erbland and E. Stotz. *J. Am. Chem. Soc.* **80**: 1624, 1958.
7. Marinetti, G. V., J. Erbland and E. Stotz. *J. Am. Chem. Soc.* **81**: 861, 1959.
8. Marinetti, G. V., J. Erbland and E. Stotz. *Biochim. et Biophys. Acta* **30**: 642, 1958.

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Time of occurrence of changes in the liver's capacity to utilize acetate for fatty acid and cholesterol synthesis after fat feeding*

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SUMMARY

The time relationships in the changes in hepatic lipogenesis and cholesterogenesis induced by the feeding of corn oil and lard have been studied. A pronounced decrease in the capacity of the liver to convert acetate carbon to fatty acids was observed as early as 1 hour after fat administration. The increase in the liver's capacity to incorporate acetate carbon into cholesterol developed slowly after fat feeding; the earliest change was observed in 12 hours. The restoration of lipogenesis to normal, following the withdrawal of fat, was more rapid than that of cholesterogenesis. The time changes in hepatic lipogenesis and cholesterogenesis induced by fat feeding were correlated with the time of appearance of exogenous fat in the liver.

Recent investigations have shown that the capacity of rat liver to utilize acetate for lipid synthesis is altered by inclusion of fat in the animal's diet. The liver's capacity to convert acetate carbon to fatty acids was measurably decreased when as little as 2.5 per cent fat was added to the diet. When 15 per cent was added, the liver retained only 10 per cent of its original capacity to convert acetate to fatty acids. Fat feeding had an opposite effect on hepatic cholesterogenesis; the presence of 15 per cent fat in the diet resulted in a three- to fourfold increase in the liver's capacity to incorporate acetate carbon into cholesterol. The fats tested were lard, corn oil, a vegetable oil, and a hydrogenated vegetable oil. They were of equal value in augmenting cholesterogenesis and in reducing lipogenesis. In the experiments under consideration, the fat content of the diet was not excessive—in no case did it exceed 15 per cent—and the period of feeding was kept short, namely, 3 days. Addition of the fats to the diets did not alter the levels of glucose, fatty acids, and cholesterol of plasma nor the glycogen, fatty acids, and cholesterol contents of the liver (1, 2).

In the present study we have examined the time relationships of the changes in hepatic lipogenesis and cholesterogenesis of rats fed fat by stomach tube. It is shown that the change in lipogenesis develops rapidly, in a matter of a few hours, but that about a day is required, after the start of fat feeding, before a measurable change in cholesterogenesis is observed.

I. Experiments with Liver Slices

EXPERIMENTAL

For 3 days before the start of the experiment, and continuing for its duration, male rats of the Long-Evans strain (200 to 250 g.) were fed, *ad libitum*, a synthetic diet containing 50 per cent glucose, 37 per cent casein (vitamin-free, Nutritional Biochemical Corp.), 6 per cent salts (3), 2 per cent defatted liver (VioBin), 5 per cent cellulose, and an adequate mixture of the B vitamins (4). Water was available to the animals at all times. At the intervals indicated by arrows in Figures 1 to 3, 2.0 ml. of corn oil or melted lard was fed by stomach tube, after which the rats were returned to their cages immediately and allowed free access to the high glucose, fat-free diet. The in-

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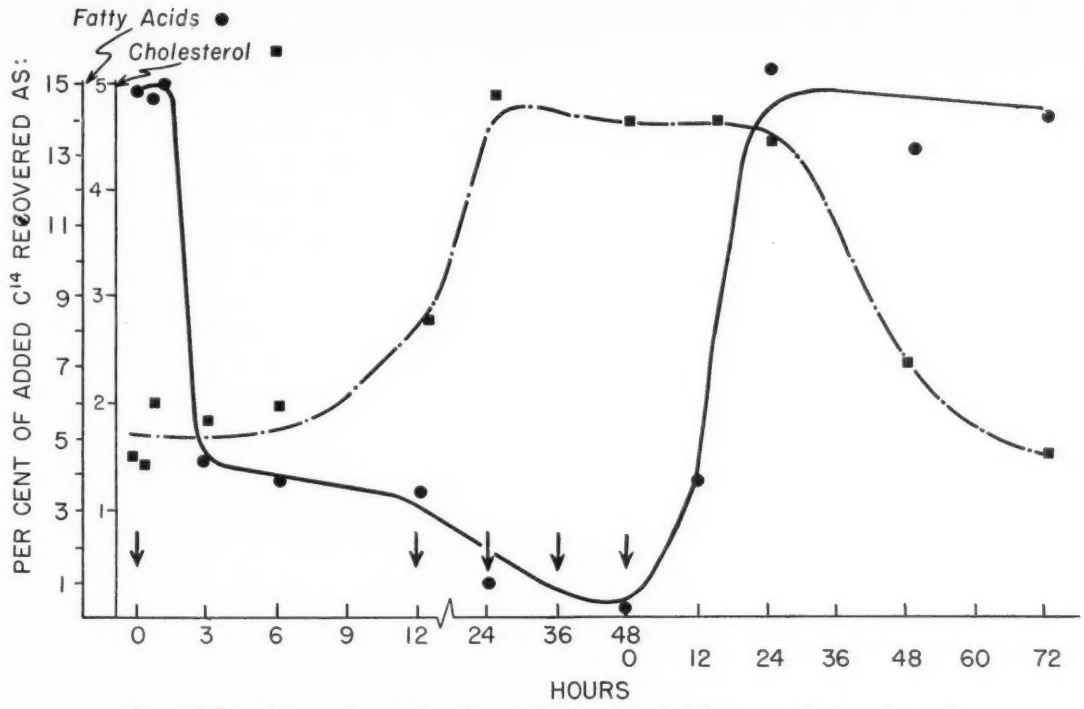

FIG. 1. Effect of ingested corn oil on hepatic lipogenesis and cholesterogenesis (experiment 1) of rats that had been fed a fat-free diet. At the times indicated by the arrows, each rat received by stomach tube 2 ml. of corn oil. Each point is the average of closely agreeing results obtained with 4 rats.

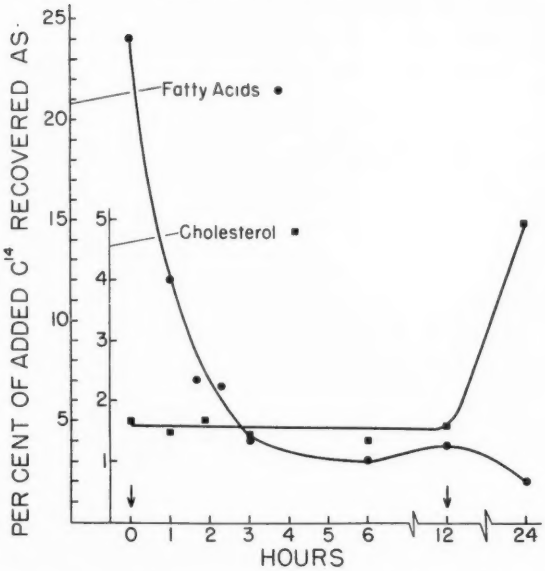

FIG. 2. Effect of ingested corn oil on hepatic lipogenesis and cholesterogenesis (experiment 2) of rats that had been fed a fat-free diet. At the times indicated by the arrows, each rat received by stomach tube 2 ml. of corn oil. Each point is the average of closely agreeing results obtained with 3 rats.

tubation of the corn oil and lard did not reduce measurably the rate of ingestion of the high glucose diet. Groups of fat-fed rats and their corresponding controls (these received 2.0 ml. of saline, instead of the fat, by intubation) were killed at various intervals thereafter. Their stomachs were examined and found to be distended with food, most of which was identified as the glucose diet. Their livers were rapidly removed, weighed, and sliced. Duplicate 500 ± 5 mg. portions were incubated, with shaking, for 3 hours at 37°C in 5.0 ml. of Krebs-Henseleit bicarbonate buffer (pH 7.3 to 7.4) (5), to which had been added 2 μmoles of acetate-1-C^{14} as the sodium salt. The methods for assaying the C^{14} content of the expired CO_2, as well as the incorporation of C^{14} into cholesterol and fatty acids, have been reported previously (1, 2).

RESULTS

The Experiments with Corn Oil. The results of two experiments with corn oil feedings are shown in Figures 1 and 2. The earliest interval after corn oil feeding at which a measurable decline was noted in the liver's capacity for incorporating acetate carbon into fatty acids was 3 hours in experiment 1 (Fig. 1) and about 1 hour in experiment 2 (Fig. 2). In experiment

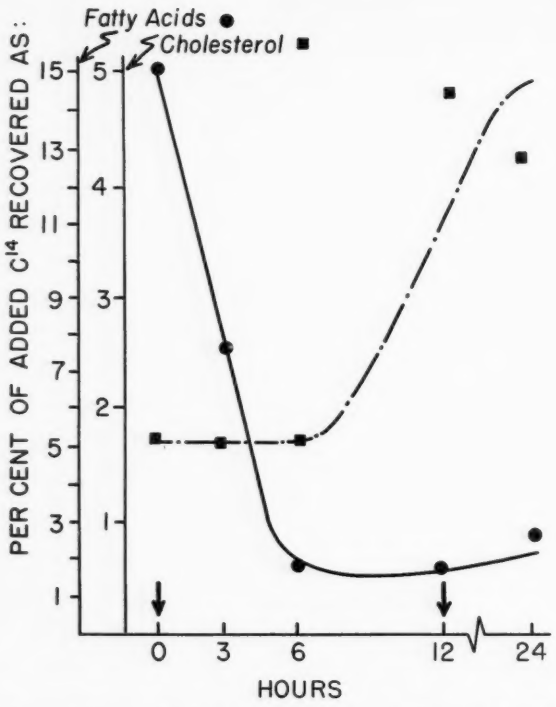

FIG. 3. Effect of ingested lard on hepatic lipogenesis and cholesterogenesis of rats that had been fed a fat-free diet. At the times indicated by the arrows, each rat received by stomach tube 2 ml. of melted lard. Each point is the average of closely agreeing results obtained with 3 rats.

1 the control rats were killed at intervals of 0.5 hour to 5 days after the first intubation of saline; in these rats the C^{14}-fatty acid recoveries (not recorded in Fig. 1) ranged from 12 to 16 per cent. At the end of 48 hours, by which time the fat-fed rats had received five administrations of corn oil, the livers had almost completely lost the capacity to form fatty acids from added acetate. The decline in C^{14}-fatty acid recoveries was also pronounced in experiment 2, which lasted 24 hours, with each rat receiving one or two feedings (2.0 ml. each) of corn oil. The C^{14}-fatty acid values found at 0 and 12 hours were 24 and 4 per cent, respectively.

No significant change in the liver's capacity to synthesize cholesterol occurred during the first 6 hours after the administration of 2.0 ml. of corn oil. A slight rise in C^{14}-cholesterol recoveries was noted at 12 hours (Fig. 1), and at 24 hours the values for C^{14}-cholesterol had more than doubled (Figs. 1 and 2).

Corn oil was last fed at the 48-hour interval (Fig. 1). Some restoration of the liver's capacity to synthesize fatty acids was observed 12 hours later. By the time 24 hours had elapsed after the last feeding, the liver had acquired its initial capacity to form fatty acids.

A delayed response in modification of the liver's capacity to synthesize cholesterol was also observed after the corn oil feedings stopped. The heightened capacity for cholesterol synthesis induced by fat feeding was still evident 24 hours after the last administration of corn oil. By 48 hours, however, the values for C^{14}-cholesterol recoveries were about the same as those observed before the corn oil feedings.

The Experiment with Lard. The results of this experiment are shown in Figure 3. The rapid loss in the liver's capacity to synthesize fatty acids and the delayed increase in its capacity for cholesterogenesis are again evident.

II. Experiments with Intact Animals

EXPERIMENTAL

Male Long-Evans rats weighing 200 to 250 g. were used. In the first experiment the feeding of the fat-free diet containing 50 per cent glucose was begun 3 days before the first administration of fat, and was continued until the rats were killed 3 and 12 hours after a single intubation of 2.0 ml. of corn oil. In the second experiment the rats were killed 72 hours after they were transferred to a diet containing 50 per cent glucose and 15 per cent corn oil. All control rats were fed the fat-free, high glucose diet throughout. Exactly 20 minutes before the rats and their respective controls were killed, they were injected intraperitoneally with 0.5 ml. of 0.9 per cent saline containing 2 μmoles of 1-C^{14}-labeled sodium acetate. The livers were rapidly excised and weighed, and aliquots were analyzed for C^{14}-fatty acids and C^{14}-cholesterol.

RESULTS

The results are recorded in Table 1. A reduction in the C^{14} incorporated into fatty acids by the livers of the intact rats was observed as early as 3 hours after the start of fat feeding, but not until more than 12 hours had elapsed were the C^{14}-cholesterol recoveries augmented. The C^{14}-cholesterol recoveries found for the livers of rats that had ingested the fat-containing diet for 72 hours were about four times those observed with the controls fed the fat-free diet.

III. Time of Appearance of Fed Fat in the Liver

The rapidity of the changes induced in hepatic lipogenesis by fat feeding raised the question of how soon

the administered fat reaches the liver. Tripalmitin-1-C^{14} was dissolved in 2.0 ml. of corn oil, and the mixture was introduced by intubation into the stomachs of rats that had been fed the fat-free, high glucose diet for 3 days. They were killed at different intervals, and their livers were excised and assayed for C^{14}-lipids. Lipids were extracted with a 3 : 1 ethanol-ether mixture, and aliquots of the extract were mounted directly on aluminum planchets. The results are shown in Figure 4. C^{14}-activity was found in the lipid fraction of the liver of rats killed as early as 1 hour after intubation. If it is assumed that the C^{14} is a measure of the transport of corn oil, it can be calculated that in 1 hour about 5 mg. of the administered oil had reached the liver, which contained about 300 mg. of fatty acids. It is of interest that the most pronounced fall in lipogenic capacity of the liver occurred at a time when so little of the administered fat had found its way into the liver. The mere presence of exogenous fat, rather than the accumulation of large amounts of it, seems to be associated with the change in hepatic lipogenesis.

DISCUSSION

Our earlier observation that fat feeding induced a change in the liver's capacity to incorporate acetate carbon into fatty acids and cholesterol is fully confirmed by the experimental findings presented here. The rapidity of the change in the lipogenic capacity of the liver, as well as the very small amounts of administered fat that reach the liver by the time the changes in lipogenic capacity are first detected, favors the idea that fatty acid synthesis in the liver is under homeostatic regulation.

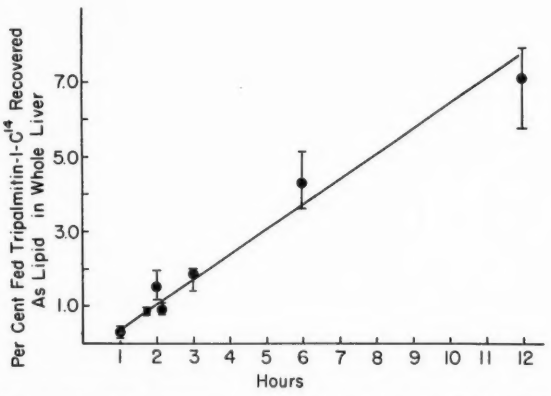

FIG. 4. Time of appearance of fed fat in the liver. Each point is the average of closely agreeing results obtained with 3 rats.

TABLE 1. EFFECT OF CORN OIL FEEDING ON INCORPORATION OF C^{14} OF ACETATE-1-C^{14} INTO FATTY ACIDS AND CHOLESTEROL BY LIVERS OF INTACT RATS

C^{14} recovered as	Hours After Start of Corn Oil Feeding		
	3	12	72
Fatty acids	−70 (−68 to −75)	−96 (−92 to −100)	−94 (−91 to −99)
Cholesterol	0 (−5 to +7)	0 (−8 to +2)	+400 (+374 to +453)

Averages and range of values obtained with 4 to 6 rats are given. They represent percentage changes from controls in the incorporation of the injected C^{14} into fatty acids and cholesterol.

The inverse effects produced by fat feeding on lipogenesis and cholesterogenesis in the liver led us to suggest earlier that cholesterogenesis, like acetoacetate formation, represents an alternate pathway for acetyl-coenzyme A when lipogenesis is depressed (6). In this case this suggestion is not borne out by the changes in cholesterogenesis and lipogenesis with time shown in Figures 1 to 4. For example, in the first experiment with liver slices, lipogenesis was depressed for more than 9 hours before a change in hepatic cholesterogenesis occurred. In the experiments with intact animals, lipogenesis was reduced for more than 12 hours before a rise in hepatic cholesterogenesis was noted.

REFERENCES

1. Hill, R., J. M. Linazasoro, F. Chevallier, and I. L. Chaikoff. *J. Biol. Chem.* **233**: 305, 1958.
2. Linazasoro, J. M., R. Hill, F. Chevallier, and I. L. Chaikoff. *J. Exptl. Med.* **107**: 813, 1958.
3. Hawk, P. B., B. L. Oser and W. H. Summerson. *Practical Physiological Chemistry*, 12th ed., Philadelphia, The Blakiston Co., 1947, p. 1273.
4. Hill, R., N. Baker and I. L. Chaikoff. *J. Biol. Chem.* **209**: 705, 1954.
5. Krebs, H. A., and K. Henseleit. *Z. physiol. Chem. Hoppe-Seyler's* **210**: 33, 1932.
6. Chaikoff, I. L., and G. W. Brown, Jr. In *Chemical Pathways of Metabolism*, edited by D. M. Greenberg, New York, Academic Press, Inc., 1954, vol. 1, p. 77.

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Oxidation of cholesterol-26-C^{14} by rat liver mitochondria: effect of nicotinic acid*

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SUMMARY

The effect of nicotinic acid upon the oxidation of the terminal carbon atoms of cholesterol-26-C^{14} by rat liver mitochondrial preparations has been studied. Addition of nicotinic acid, as the potassium salt, to incubation mixtures containing normal rat liver mitochondria enhances the oxidation of cholesterol-26-C^{14}. Liver mitochondrial preparations from rats administered nicotinic acid in drinking water (12 mg. per day) or diet (0.75 per cent) also oxidize cholesterol-26-C^{14} to a greater extent than do similar preparations from control rats. The mitochondrial preparations from nicotinic acid-fed rats, in the absence of boiled supernatant, exhibit a greater oxidative capacity than do control preparations. This heightened oxidative capacity is further enhanced by addition of the boiled supernatant fraction from these preparations. Addition of boiled supernatant from control preparations has no enhancing effect. Nicotinic acid exerts little effect on the oxidation of sodium pyruvate-2-C^{14} and may have a slightly inhibitory effect upon the oxidation of sodium octanoate-1-C^{14}. No significant differences were observed between serum cholesterol levels of rats ingesting nicotinic acid for 3 weeks and controls.

Since the initial report on the hypocholesterolemic effect of nicotinic acid administered orally to humans (1), a large number of confirmatory publications have appeared (2). Nicotinic acid has been shown to lower serum cholesterol levels in hypercholesterolemic rabbits (3, 4) and dogs (5), although one group of investigators (6) could not confirm the latter result. Nicotinic acid also lessens the degree of aortic atheroma when administered to cholesterol-fed rabbits (3, 4).

The mechanism by which nicotinic acid exerts its hypocholesterolemic effect is not yet known. The feeding of this compound to cholesterol-fed rabbits (4) or rats (7) lowers liver cholesterol levels as compared with cholesterol-fed controls. There is no reduction to normal levels, however. Feeding of nicotinic acid to normal rats (1 to 2 per cent in diet) causes increases in liver fat (8) and liver cholesterol (9) content. Nicotinic acid has also been found to depress cholesterol synthesis from acetate by one group of workers (9) and to enhance it by another (10). Suggestions that the nicotinic acid effect involves the pyridine nucleotides which play an important role in steroid oxidation (11) are weakened by findings that nicotinamide has no hypocholesterolemic effect (10, 12). Hoffer *et al.* (13) have found that beta-pyridylcarbinol is also ineffective, suggesting a specific structural requirement, namely, a free carboxyl group. Nicotinic acid feeding causes a decrease in urinary glycine with increased excretion of nicotinuric acid (10, 14); the latter compound is under test (13). The reduced glycine output might also be due to increased formation of glycocholic acid, but no data are available on this point. Altschul originally suggested that nicotinic acid may act by increasing the *in vivo* oxidation of cholesterol (1).

In the present study we have investigated the effect of nicotinic acid upon the oxidation of the terminal carbon atoms of cholesterol-26-C^{14} by rat liver mitochondrial preparations. In one group of experiments the nicotinic acid (as potassium nicotinate) was added directly to the mitochondrial preparation. In other

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TABLE 1. EFFECT OF ADDITION OF NICOTINIC ACID ON OXIDATION OF CHOLESTEROL-26-C^{14} BY RAT LIVER MITOCHONDRIA

Experiment	Nicotinic Acid †	Percentage of Oxidation of Cholesterol *			
		Males		Females	
		Nicotinic Acid	Control	Nicotinic Acid	Control
	mg.				
1	6	20.5	8.8	12.3	9.2
2	15	9.4	6.0	37.5	31.6
3	3	7.9			
	6	8.2	9.0		
	12	9.2			
4	6	5.6	4.2	6.2	5.4
5	6	2.4	1.9	4.3	4.2
6	6	6.2	2.2	3.4	2.8
7	6	4.5	3.3	6.7	7.1
8	6	5.0	4.4	25.5	22.5

* Computed as $BaC^{14}O_3$/cholesterol-26-C^{14} × 100.
† Added to incubation mixture as potassium salt.

experiments the mitochondrial preparations were obtained from rats which had previously been fed nicotinic acid, either as the sodium salt in the drinking water or as the free acid in the food. Salts of nicotinic acid are as efficacious as the free acid (15).

METHODS

The procedures for obtaining the mitochondrial preparations and supernatant fractions used in these experiments have been described in detail elsewhere (16, 17). It was noted that after the initial low-speed centrifugation the cellular debris from the livers of rats fed nicotinic acid was arranged in layers with sharp boundaries between the various fractions. This phenomenon had not been observed with livers of rats used in other experiments.

The incubation was carried out in stoppered 125 ml. Erlenmeyer flasks containing center wells. The percentage oxidation of cholesterol-26-C^{14}, sodium pyruvate-2-C^{14}, and sodium octanoate-1-C^{14} was calculated from analysis of the $C^{14}O_2$ evolved during the incubation.

The incubation mixture consisted of 1 ml. of the mitochondrial preparation; 1 ml. of a solution containing adenosine triphosphate (25 mg.), diphosphopyridine nucleotide (5 mg.), adenosine monophosphate (8 mg.), reduced glutathione (15 mg.), sodium citrate monohydrate (30 mg.), magnesium nitrate hexahydrate (10 mg.), potassium penicillin G (2,000 units), and streptomycin sulfate (1 mg.); 5 ml. of labeled substrate emulsion in 0.25M tris (hydroxymethyl) aminomethane-HCl, pH 8.5; and boiled supernatant fluid (5 ml.).

The soluble cofactor was prepared by boiling the liver supernatant after centrifugation at 104,000 × *g* for 30 minutes.

The labeled cholesterol was synthesized from 3 β-hydoxy-Δ^5-norcholesten-25-one by the method of Dauben and Bradlow (18). Sodium pyruvate-2-C^{14} and octanoic acid-1-C^{14} were purchased from the Atomic Energy Research Establishment, Amersham, England. The cholesterol suspension was prepared in the manner previously described (17). The acids were dissolved directly in the buffer. All cholesterol determinations were carried out using the method of Trinder (19).

Animals used in the first two series of experiments were maintained on Purina Laboratory Chow. The nicotinic acid diet contained (per 100 g.): Pablum, 70 g.; wheat germ, 6.25 g.; skim milk powder, 20 g.; Vionate, 3 g.; and nicotinic acid, 0.75 g.[1] Sufficient water was added to make a thick dough. The control

TABLE 2. EFFECT OF ADDITION OF NICOTINIC ACID ON OXIDATION OF SODIUM PYRUVATE-2-C^{14} BY RAT LIVER MITOCHONDRIA

Experiment	Nicotinic Acid *	Percentage of Oxidation of Pyruvate			
		Males		Females	
		Nicotinic Acid	Control	Nicotinic Acid	Control
	mg.				
1	6	3.1	1.4	4.4	5.2
2	15	11.0	9.2	24.5	26.8
3	3	9.2			
	6	10.1	3.8	15.3	20.5
	12	8.3			
4	6	2.3	1.9		
5	6	1.3	1.4		
6	6	2.1	1.5		

* Added to incubation mixture as potassium salt.

[1] Pablum: Mead Johnson & Co., Evansville 21, Ind.; wheat germ: Kretschmer Corp., Carrollton, Mich.; skim milk powder: Land O'Lakes Creameries, Inc., Minneapolis, Minn.; Vionate: E. R. Squibb & Sons, New York 22, N. Y.; nicotinic acid: Matheson, Coleman & Bell Division, East Rutherford, N. J.

TABLE 3. AUTOPSY DATA* ON RATS GIVEN NICOTINIC ACID (12 MG./DAY) IN THEIR DRINKING WATER

Sex	Drinking Water	Number of Rats	Final Weight	Weight Change	Liver Weight	Serum Cholesterol
			g.	*g.*	*g.*	*mg./100 ml.*
Male	Nicotinic acid	3	330	+33	10.85	49.3 ± 3.2 †
	Control	5	293	+25	9.99	54.3 ± 5.8
Female	Nicotinic acid	3	251	+22	9.98	48.6 ± 4.7
	Control	5	237	+23	8.88	58 2 ± 4.2

* Averages.
† Standard error of the mean.

rats were fed the same diet minus nicotinic acid. No significant difference in food consumption between the nicotinic acid and control groups was noted. This diet provides 20 per cent protein, 11 per cent fat, and 62 per cent carbohydrate. It is well received by the animals used in these and other experiments.

Experiments using nicotinic acid in the food were carried out with Wistar rats, both sexes, weighing 150 to 160 g. The diet was administered for 3 weeks prior to the experiment. Experiments using sodium nicotinate in the drinking water were carried out with male Wistar rats weighing 260 to 300 g. These animals were maintained on the sodium nicotinate (12 mg. per day) for 3 weeks.

Each experiment was carried out using mitochondria from the same rat. In experiment 3, Table 1, for example, all four incubations (three nicotinic acid, one control) were carried out using aliquots of the same mitochondrial preparation. Preliminary experiments showed that aliquots of the same preparation consistently oxidized cholesterol to the same extent. In one specific example, the range of oxidation with the same mitochondrial preparation varied between 5.7 and 6.3 per cent in a series of six incubations.

RESULTS AND DISCUSSION

Addition of Nicotinic Acid to Normal Mitochondrial Preparations. Experiments were carried out using preparations from both male and female rat livers. The data on oxidation of cholesterol are given in Table 1 and on pyruvate oxidation in Table 2.

In practically all cases the addition of nicotinic acid to the incubation mixture enhanced oxidation of cholesterol-26-C^{14}. Only in experiment 3 (males) and 7 (females) was there not some enhancement of oxidation. As has been noted previously (17), normal female rat liver mitochondria appear to have the greater oxidative capacity for cholesterol-26-C^{14}. The oxidation of pyruvate-2-C^{14} by male rat liver mitochondria is also enhanced by the addition of nicotinic acid, but no such effect is evident in the case of female rat liver preparations. The much greater oxidation of pyruvate by female rat liver mitochondria is noteworthy. This phenomenon, too, has been pointed out before (17).

In one experiment where sodium octanoate-1-C^{14} was the substrate, oxidation was not enhanced by addition of nicotinic acid to the medium (24.3 vs. 24.3

TABLE 4. PER CENT OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM PYRUVATE-2-C^{14} BY LIVER MITOCHONDRIA OF RATS GIVEN NICOTINIC ACID (12 MG./DAY) IN THEIR DRINKING WATER

Experiment	Drinking Water	Percentage of Oxidation of Substrates *			
		Males		Females	
		Cholesterol	Pyruvate	Cholesterol	Pyruvate
1	Nicotinic acid	25.8	8.3	lost	lost
	Control	8.8	1.4	5.2	9.2
2	Nicotinic acid	6.6	5.2	19.3	22.1
	Control	6.0	9.2	26.8	31.6
3	Nicotinic acid	22.2	20.3	14.7	14.0
	Control	14.0	14.5	18.6	21.7

* All values corrected for equivalent amounts of mitochondria (mg. N).

TABLE 5. AUTOPSY DATA* ON RATS FED NICOTINIC ACID (0.75% OF DIET)

Sex	Diet	Number of Rats	Final Weight	Weight Change	Liver Weight	Serum Cholesterol
			g.	*g.*	*g.*	*mg./100 ml.*
Male	Nicotinic acid	7	198	48	7.56	60.1 ± 3.4 †
	Control	8	201	49	8.52	62.6 ± 1.9
Female	Nicotinic acid	5	180	24	7.88	63.4 ± 6.9
	Control	7	170	21	6.65	57.1 ± 6.8

* Averages.
† Standard error of the mean.

per cent for the control preparation in the male and 19.0 vs. 25.1 per cent for the control in the female).

Experiments were also carried out in which picolinic acid and pyridine-3-sulfonic acid were added as the sodium salts to various incubation mixtures. The results were variable, but generally a slight enhancement of cholesterol oxidation was observed. Nicotinamide added at levels up to 30 mg. per incubation failed to enhance cholesterol and pyruvate oxidation by normal rat liver mitochondria.

Effect of Nicotinic Acid in Drinking Water. The administration of 12 mg. of nicotinic acid per day in the drinking water was carried out after first establishing that the animals used in these experiments took 2 days to empty a 240 ml. container. We administered fresh water containing sodium nicotinate (0.1 mg. per ml.) daily.

Autopsy data on these animals are presented in Table 3. There was a slight, but nonsignificant, lowering of serum cholesterol levels in both males and females administered nicotinic acid. The results of the oxidation of cholesterol-26-C^{14} and of sodium pyruvate-2-C^{14} are presented in Table 4. There was little doubt about the increased cholesterol oxidation by male liver mitochondria in the nicotinic acid group. Liver mitochondrial preparations from the female control rats oxidized more of both substrates than did the experimental group. The nicotinic acid may not exert a great effect here because of the higher oxidative capacity (as compared with males).

Effect of Nicotinic Acid in Diet. The autopsy data are presented in Table 5. The serum cholesterol differences (higher in male controls and female nicotinic groups) are not significant. Data on the oxidation of various substrates by liver mitochondrial preparations taken from these animals are given in Table 6. Again we find an increase in cholesterol oxidation, slight effect on pyruvate oxidation, and actual reduction of oxidation of octanoate by liver preparations from nicotinic acid-fed rats. On two occasions we added 6 mg. of nicotinic acid (as the potassium salt) to incubation mixtures containing mitochondria from nicotinic acid-fed rats and found a further enhancement of cholesterol oxidation (8.4 vs. 7.1 per cent and 17.7 vs. 14.9 per cent). There was no effect on pyruvate oxidation; if anything, there was a slight reduction (1.5 vs. 1.7 per cent). These results indicate that we had probably not reached the maximum level in the dietary dosage.

In an earlier experiment (17) we had observed appreciable changes in extent of oxidation of choles-

TABLE 6. PER CENT OXIDATION OF CHOLESTEROL-26-C^{14} SODIUM PYRUVATE-2-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY LIVER MITOCHONDRIA FROM RATS FED NICOTINIC ACID (0.75% IN DIET)

Substrate	Experiment	Percentage of Oxidation of Substrates *			
		Males		Females	
		Nicotinic acid	Control	Nicotinic acid	Control
Cholesterol	1	7.1	1.9	34.8	22.5
	2	14.9	2.2	15.0	13.9
	3	17.7	4.4	6.5	2.9
	4	5.7	5.3		
	5	2.1	2.1		
Pyruvate	1	3.1	1.9		
	2	1.7	1.4		
Octanoate	1	13.9	24.3	21.5	25.1
	2	20.5	26.8	30.2	35.6

* All values corrected for equivalent amounts of mitochondria (mg. N).

TABLE 7. EFFECT OF SUBSTITUTION OF SUPERNATANT ON OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY RAT LIVER MITOCHONDRIA

Mitochondria	Supernatant	Percentage of Oxidation of Substrates *			
		Males		Females	
		Cholesterol	Octanoate	Cholesterol	Octanoate
Nicotinic	None †	13.2	13.9	28.2	27.6
Nicotinic	Nicotinic acid	17.7	13.9	34.8	21.5
Nicotinic	Control	12.7	16.5	27.2	27.1
Control	None	3.6	25.5	15.4	24.9
Control	Nicotinic acid	7.8	25.3	25.0	23.8
Control	Control	4.4	24.3	22.5	25.1

* All values corrected for equivalent amounts of mitochondria (mg. N).

† Equivalent amount of 10% sucrose used.

terol when the boiled supernatant fraction from one dietary group was added to the mitochondria of another. This type of "crossover" experiment was repeated in the present series with results as shown in Table 7. It is evident that the supernatant fraction from the nicotinic acid-fed rat liver preparation has an enhancing property for cholesterol oxidation. In the case of octanoate this supernatant is inhibitory, if anything. The data also suggest that in the absence of "nicotinic" supernatant the "nicotinic" mitochondrial preparation has considerably more oxidative potential than does that from the control rat liver. Thus, a factor within the mitochondria is responsible for most of the heightened oxidative capacity in preparations from livers of nicotinic acid-fed rats, but this effect can be increased by the addition of boiled supernatant from the same preparation.

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REFERENCES

1. Altschul, R., A. Hoffer and J. D. Stephen. *Arch. Biochem. Biophys.* **54**: 558, 1955.
2. Portman, O. W., and F. J. Stare. *Physiol. Revs.* **39**: 407, 1959.
3. Altschul, R. *Z. Kreislaufforsch.* **45**: 573, 1956.
4. Merrill, J. M., and J. Lemley-Stone. *Circulation Research* **5**: 617, 1957.
5. Nava, A., F. Comesaña, E. Lozano, B. L. Fishleder, and D. Sodi-Pallares. *Am. Heart J.* **56**: 598, 1958.
6. Norcia, L. N., H. J. Brown and R. H. Furman. *Lancet* **1**: 1255, 1959.
7. Schön, H. *Nature* **182**: 534, 1958.
8. Handler, P., and W. J. Dann. *J. Biol. Chem.* **146**: 357, 1942.
9. Duncan, C. H., and M. M. Best. *Circulation* **18**: 490, 1958.
10. Hoffer, A., and M. J. Callbeck. *J. Mental Sci.* **103**: 810, 1957.
11. Merrill, J. M. *Circulation Research* **6**: 482, 1958.
12. Parsons, W. B., Jr., and J. H. Flinn. *Circulation* **18**: 489, 1958.
13. Hoffer, A., P. O. O'Reilly and M. J. Callbeck. *Diseases of Nervous System* **20**: 286, 1959.
14. Miller, O. N., J. G. Hamilton and G. A. Goldsmith. *Circulation* **18**: 489, 1958.
15. Altschul, R., and A. Hoffer. *Brit. Med. J.* **2**: 713, 1958.
16. Whitehouse, M. W., E. Staple and S. Gurin. *J. Biol. Chem.* **234**: 276, 1959.
17. Kritchevsky, D., R. R. Kolman, M. W. Whitehouse, M. C. Cottrell, and E. Staple. *J. Lipid Research* **1**: 83, 1959.
18. Dauben, W. G., and H. L. Bradlow. *J. Am. Chem. Soc.* **72**: 4248, 1950.
19. Trinder, P. *Analyst* **77**: 321, 1952.

Volume 1
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Lack of nicotinic acid effect on cholesterol metabolism of the rat*

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SUMMARY

The effect of the addition of 1 per cent nicotinic acid to the diet of the rat on serum, liver, and carcass cholesterol, and on the incorporation of acetate-1-C^{14} into cholesterol by the intact animal were studied. The effect of nicotinic acid on absorption of cholesterol-4-C^{14} and on the elimination of labeled cholesterol from serum and liver were also studied. No significant effect of nicotinic acid, fed for either 8 or 42 days, on the incorporation of acetate-1-C^{14} into serum and liver cholesterol was observed. When weight gain of the control animals was limited to that of the nicotinic-acid-fed rats by pair-feeding, nicotinic acid had no significant effect on serum or liver cholesterol concentrations, nor on total carcass cholesterol. Absorption of cholesterol-4-C^{14} and the disappearance of labeled cholesterol from serum and liver were not influenced by nicotinic acid administration by stomach tube.

The addition of nicotinic acid to the diet for 8 days has been reported by Merrill to result in an increased incorporation of acetate-1-C^{14} into liver cholesterol by the rat (1). In a preliminary report we were unable to confirm this finding, the addition of 1 per cent nicotinic acid to the diet for 8 days having no effect on the incorporation of acetate into serum and liver cholesterol by the intact rat. After 42 days the mean incorporation of acetate into cholesterol was less in the nicotinic-acid-fed animals than in the controls, although the difference was not statistically significant (2).

In view of the widespread interest in nicotinic acid as a hypocholesterolemic agent in man (3, 4, 5), its effect on cholesterol metabolism of the rat has been further studied. Data have been obtained regarding its effect on the absorption and elimination of cholesterol as well as on the incorporation of acetate-1-C^{14} into cholesterol by the intact animal.

METHODS

Male albino rats (Holtzman) were employed in all studies, being housed in mesh-bottomed cages and kept in an air-conditioned animal room. Tap water and a balanced laboratory mash,[1] to which cottonseed oil, 5 ml. per 100 g., was added, were offered *ad libitum* except as noted below. When nicotinic acid was fed, it was incorporated into the diet in the amount of 1 per cent by weight.

The animals were killed under amobarbital sodium anesthesia, blood obtained by cardiotomy, and the livers removed, blotted, and weighed. Calculations were based on an assumed plasma volume of 3.42 per cent of body weight.

Serum cholesterol was determined by the method of Abell *et al.* (6). A 2-to-3 g. segment of liver was digested by refluxing for 2 hours in 10 per cent potassium hydroxide in 70 per cent ethanol. After neutralization with acetic acid and dilution to volume, an aliquot of the liver digest was taken for determination of total cholesterol by the method of Sperry and Webb (7).

Serum cholesterol was prepared for liquid scintillation counting of C^{14} as follows: Two ml. of serum was mixed with 8 ml. 95 per cent ethanol containing 2 per cent (w/v) potassium hydroxide and incubated at 37°C for 55 minutes. After cooling to room temperature, 5 ml. of distilled water was added and the mixture extracted four times with 5 ml. portions of

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[1] Rat-Mouse Ration, Harlan Small Animal Industries, Cumberland, Ind.

TABLE 1. INCORPORATION OF SODIUM ACETATE-1-C^{14} INTO SERUM AND LIVER CHOLESTEROL*

Diet	Experimental Period	Number of Rats	Initial Weight	Weight Gain	Serum Cholesterol	Liver Cholesterol	Incorporation of C^{14} into Cholesterol
	no. of days		*g.*	*g.*	*mg./100 ml.*	*mg./100 g.*	*percentage*
Control	8	8	288	35 ± 9	68 ± 7	251 ± 21	.151 ± .036
Nicotinic acid		8	288	24 ± 10 †	65 ± 6	251 ± 12	.163 ± .073
Control	42	4	269	137 ± 14	68 ± 3	256 ± 5	.174 ± .025
Nicotinic acid		4	266	92 ± 26 †	60 ± 4 †	282 ± 24	.123 ± .038
Control	42‡	7	361	32 ± 13	67 ± 10	272 ± 42	.227 ± .026
Nicotinic acid		7	361	32 ± 11	65 ± 9	295 ± 55	.219 ± .018

* Values given are the mean and standard deviation, estimated using (n − 1).
† Values that differ significantly from the Control ($p < .05$).
‡ Paired weight gain.

TABLE 2. ABSORPTION OF CHOLESTEROL-4-C^{14}*

Gavage Solution	Number of Rats	Weight	Serum Cholesterol	Liver Cholesterol	Administered C^{14} in Serum and Liver Cholesterol
		g.	*mg./100 ml.*	*mg./100 g.*	*percentage*
Control	6	321	55 ± 6	198 ± 23	4.83 ± 1.28
Nicotinic acid	6	316	54 ± 5	220 ± 39	5.77 ± 1.19

*Values given are the mean and standard deviation, estimated using (n − 1).

petroleum ether. The combined petroleum ether extracts were evaporated to dryness under nitrogen. The residue was redissolved in 1:1 acetone-ethanol, acidified with acetic acid, filtered, digitonin added, an estimated 50 per cent excess being employed, and the tube allowed to stand overnight at room temperature. After washing with 1:2 acetone ether and then ether, the cholesterol digitonide was dissolved in 10 ml. of methanol, 10 ml. of toluene added, and the digitonide decomposed by evaporation to dryness with heat and a stream of nitrogen. The residue was then redissolved in 14 ml. of toluene, two 1.5 ml. aliquots taken for duplicate determination of cholesterol, and 9 ml. added to the 20 ml. counting vial containing 30 mg. of the scintillator, 2,5-diphenyloxazole, in 1 ml. of toluene.

An aliquot of the neutralized liver digest, representing approximately 1 g. of liver, was extracted with petroleum ether, and the cholesterol prepared for counting in the same manner as described for serum cholesterol. In the Packard Tri-Carb liquid scintillation counter the background was approximately 8 cpm. and the counting efficiency, determined with a C^{14}-benzoic acid standard, 44 per cent.

Incorporation of Sodium Acetate-1-C^{14} into Serum and Liver Cholesterol. Control and 1 per cent nicotinic acid diets were fed *ad libitum* to groups of rats for 8 and 42 days. Two hours before sacrifice each rat received an intraperitoneal injection of carboxyl labeled sodium acetate, 2 μc. per 100 g. body weight. Labeled acetate with a specific activity of 25 μc. per mmole was used, each animal receiving 25 to 30 mg. of sodium acetate in aqueous solution.

The 42-day feeding of nicotinic acid was also carried out, using seven pairs of rats housed in individual cages. The rats given nicotinic acid were fed *ad libitum* and weighed every other day; the control rats were given just sufficient food to maintain the same weight gain as the paired experimental animals. In addition

TABLE 3. DISAPPEARANCE OF CHOLESTEROL-C^{14} FROM SERUM AND LIVER *

Group	Number of Rats	Initial Weight	Serum Cholesterol	Liver Cholesterol	Administered C^{14} in Serum and Liver Cholesterol
		g.	*mg./100 ml.*	*mg./100 g.*	*percentage*
2 hours after mevalonic acid administration	4	369	53 ± 5	224 ± 13	5.14 ± 1.19
6 days after mevalonic acid administration: control gavage†	4	359	64 ± 7	218 ± 12	.679 ± .066
6 days after mevalonic acid administration: nicotinic acid gavage†	4	348	65 ± 6	228 ± 11	.684 ± .227

* Values given are the mean and standard deviation, estimated using (n − 1).
† Third through sixth day.

to the other observations, the oxygen consumption of each of these rats was determined in a closed-circuit respirometer, and at sacrifice the thyroid gland was weighed (8).

Cholesterol Absorption. After fasting overnight, each of 12 rats was fed by gavage with 1 ml. of cottonseed oil in which was dissolved 10 mg. of cholesterol containing 0.8 μc. of cholesterol-4-C^{14}. At the same time six of the animals also received by gavage a solution of sodium nicotinate equivalent to 65 mg. of nicotinic acid. The remaining six were given an equal volume of water by gavage. The basic laboratory diet and water were offered *ad libitum* until the animals were killed 24 hours later, and cholesterol-C^{14} content of serum and liver determined.

Cholesterol Excretion. The cholesterol of 12 rats was labeled by the intraperitoneal injection of mevalonic acid-2-C^{14}, 0.1 μc. per 100 g. body weight. Two hours later four rats were killed and the incorporation of C^{14} into serum and liver cholesterol determined. The remaining animals were divided into two groups, each receiving the basic diet. From the third through the sixth day the rats in one group were given sodium nicotinate equivalent to 25 mg. nicotinic acid per 100 g. body weight twice daily by gavage; the remaining animals received a like volume of water by gavage. The animals were then killed and the C^{14} remaining in serum and liver cholesterol determined.

Total Rat Cholesterol. Two groups of four rats were maintained for 42 days, one on 1 per cent nicotinic acid diet *ad libitum*, and the other on the basic diet, weight gain of paired animals being controlled as previously described. Serum and liver cholesterol were determined in the usual manner. In addition, the residual carcass, including the gastrointestinal tract, from which the contents had been removed by irrigation, was digested by refluxing overnight in alcoholic potassium hydroxide, and total carcass cholesterol determined by the same method used for liver cholesterol.

RESULTS

Incorporation of Sodium Acetate-1-C^{14} into Serum and Liver Cholesterol. The addition of 1 per cent nicotinic acid to the *ad libitum* diet resulted in a significant inhibition of weight gain, apparent in both the 8- and the 42-day experiments (Table 1). A modest but statistically significant reduction of serum cholesterol was noted in the nicotinic-acid-fed animals receiving the *ad libitum* diets for 42 days. The most pronounced difference between these animals and their control group is in the partition of cholesterol between serum and liver, the ratio of liver cholesterol (mg. per 100 g.) to serum cholesterol (mg. per 100 ml.) being 3.79 ± .21 in the control group and 4.72 ± .13 in the nicotinic-acid-fed group ($p < .01$).

In the final experiment, in which the weight gain of the control group was restricted to that of the nicotinic-acid-fed group, no significant effect of nicotinic acid on serum or liver cholesterol was observed. Nor was any significant effect of nicotinic acid on oxygen consumption observed, the mean oxygen consumption of the seven control animals being 27.4 ± 4.0 ml. per 10 minutes per 100 g. to the two-thirds power, and that of the nicotinic-acid-fed group, 28.5 ± 2.9. Thyroid weights were also essentially identical, 4.8 ± .6 mg. per 100 g. body weight in the control group and 4.7 ± .7 in the nicotinic-acid-fed group.

TABLE 4. TOTAL CHOLESTEROL CONTENT OF SERUM, LIVER, AND RESIDUAL CARCASS. 42-DAY EXPERIMENTAL PERIOD—PAIRED WEIGHT GAIN *

Diet	Number of Rats	Initial Weight	Weight Gain	Total Cholesterol Content			
				Serum	Liver	Carcass	Total
		g.	*g.*	*mg.*	*mg.*	*mg.*	*mg.*
Control	4	281	103 ± 9	8.7 ± 1.3	23.5 ± 2.6	501 ± 56	533.2 ± 57.9
Nicotinic acid	4	284	103 ± 2	9.0 ± 1.7	25.4 ± 2.1	508 ± 37	542.4 ± 40.2

* Values given are the mean and standard deviation, estimated using (n −1).

In none of the experiments did nicotinic acid significantly influence the incorporation of sodium acetate-1-C^{14} into serum and liver cholesterol.

Cholesterol Absorption. Twenty-four hours after the administration of cholesterol-4-C^{14} approximately 5 per cent of that administered was present in serum and liver (Table 2). There was no significant difference between the control and nicotinic acid groups.

Cholesterol Excretion. The incorporation of mevalonic acid-2-C^{14} into cholesterol was much more efficient than that of acetate, approximately 5 per cent of the administered C^{14} being present in serum and liver cholesterol 2 hours after its injection (Table 3). Six days later essentially identical amounts of labeled cholesterol remained in serum and liver of the control and nicotinic-acid-treated rats.

Total Rat Cholesterol. As compared to their weight gain paired controls, the four rats fed 1 per cent nicotinic acid for 42 days displayed no significant difference in total cholesterol content or in its distribution between serum, liver, and residual carcass (Table 4).

DISCUSSION

The addition of 1 per cent nicotinic acid to a balanced laboratory mash consistently exerted an inhibitory effect on weight gain. Although only an approximate measure of daily food intake was made, it was apparent that the nicotinic-acid-fed animals consumed less than the controls, and it is assumed that the decreased rate of growth was a consequence of decreased food intake. That it was not due to hypermetabolism is suggested by the results of the oxygen consumption measurements, the mean oxygen consumption of the nicotinic-acid-fed rats not differing significantly from that of the controls. This finding in the rat is in disagreement with the reported increase in basal metabolic rate of the human produced by nicotinic acid (5, 9).

As previously reported in a larger series (2), the mean serum cholesterol of rats fed nicotinic acid for 42 days was slightly but significantly lower than that of controls maintained on *ad libitum* diet. However, when the food intake of the controls was restricted so that their rate of weight gain was the same as that of the nicotinic-acid-fed animals, there was no significant difference in serum cholesterol levels. It would thus appear that in the rat the hypocholesterolemic effect of nicotinic acid is due at least in large part to its anorectic effect. That the value which best differentiates the nicotinic-acid-fed animals from their controls is the ratio of liver to serum cholesterol also suggests that the reduced serum cholesterol is the result of a shift of the plasma-liver pool of cholesterol from plasma to liver, rather than any net change in cholesterol balance.

No consistent effect of nicotinic acid on the incorporation of acetate-1-C^{14} into cholesterol was observed. The reason for the failure to observe the increased incorporation in the nicotinic-acid-fed groups reported by Merrill is not apparent (1). Since he reported only relative values for incorporation, exact comparison of the data is not possible.

The greater incorporation of acetate into cholesterol in both control and nicotinic-acid-fed animals in the experiment with paired weight gain may be the result of either their greater age and weight or of a difference in the experimental design. In the groups allowed *ad libitum* diet, no period of fasting was imposed, and the acetate was injected between 11:00 A.M. and noon. Because the control rats on restricted food intake consumed all their food by early evening, while the nicotinic-acid-fed rats continued to eat during the night, it was necessary to amend this procedure to avoid differences in acetate incorporation into cholesterol due to differences in immediate nutritional state of the animals. Both groups were fasted overnight prior to acetate injection. At 8:00 A.M. each animal

was given 5 g. of food, all of which was consumed by both control and nicotinic-acid-fed rats within 1 hour. Acetate was then injected 2 to 3 hours later.

With regard to the absorption of cholesterol, no acute effect of nicotinic acid was observed. Nor did the administration of nicotinic acid for 4 days have any effect on the disappearance of cholesterol-C^{14} from serum and liver. Assuming that the disappearance of tagged cholesterol from serum and liver is a valid reflection of disappearance of cholesterol from all the other body pools, this indicates a lack of nicotinic acid effect on cholesterol excretion.

That the more prolonged ingestion of nicotinic acid (42 days) has no measurable net effect on cholesterol balance in the rat, providing weight gain of experimental and control animals is matched, is indicated by the final experiment.

The hypocholesterolemic effect of nicotinic acid in man cannot be due solely to any effect it may have on quantity or type of food selected, since Goldsmith *et al.* have observed it to effect a lowering of serum cholesterol in patients maintained on constant diets (10). It thus seems that the rat differs from man in its lack of response to nicotinic acid, provided diet is controlled.

REFERENCES

1. Merrill, J. M. *Circulation Research* **6**: 482, 1958.
2. Duncan, C. H., and M. M. Best. *Circulation* **18**: 490, 1958.
3. Altschul, R., A. Hoffer and J. D. Stephen. *Arch Biochem. Biophys.* **54**: 558, 1955.
4. Parsons, W. B., Jr., and J. H. Flinn. *A.M.A. Arch. Internal Med.* **103**: 122, 1959.
5. Gurian, H., and D. Adlersberg. *Am. J. Med. Sci.* **237**: 12, 1959.
6. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195**: 357, 1952.
7. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187**: 97, 1950.
8. Best, M. M., and C. H. Duncan. *Am. J. Physiol.* **196**: 857, 1959.
9. Altschul, R., and A. Hoffer. *Arch. Biochem. Biophys.* **73**: 420, 1958.
10. Goldsmith, G. A., J. G. Hamilton and O. N. Miller. *Federation Proc.* **18**: 526, 1959.

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Effects of intravenous heparin on oxidation of fat

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SUMMARY

The intravenous injection of heparin to carbohydrate-fed rats results in an elevation of plasma free fatty acid levels and in a lowering of the respiratory quotient from 1.0 to 0.9. The injection of heparin simultaneously with palmitic acid-C^{14}-labeled chylomicrons results in an elevation of the specific radioactivity of the expired CO_2. These observations suggest that heparin causes an obligatory oxidation of fat, presumably through the release of free fatty acid from triglyceride.

It has been repeatedly observed in a variety of mammalian species, including man, that the intravenous injection of heparin is followed immediately by the appearance in the blood stream of a "clearing factor." This factor is apparently identical with the enzyme "lipoprotein lipase" isolated by Korn and Quigley (1) from adipose tissue. It characteristically hydrolyzes the triglycerides contained in chylomicrons and low-density lipoproteins with the accumulation, at least *in vitro*, of free fatty acids (FFA). In plasma, the latter are "free" in the sense that they are not in covalent linkage, but they are, for the most part, tightly bound to albumin.

It has been demonstrated by several groups (2, 3) that FFA leave the blood stream more rapidly than do chylomicrons, although the half life of the latter is a matter of only a few minutes, dependent to some extent on the dose (4). If it is assumed that the first step in the oxidation of triglycerides is a hydrolytic one to form the free fatty acids, then one might expect the injection of heparin to have some effect on the oxidation of triglyceride fat.

French and Morris (5) have already demonstrated in fed rats injected with palmitic acid-C^{14}-labeled chyle that heparin did indeed increase the amount of $C^{14}O_2$ excreted, particularly in the first hour. They did not determine the respiratory quotient. The present experiments were designed to confirm the findings of French and Morris and to extend them.

METHODS

Two different types of experiment were designed to elucidate the role of heparin in the oxidation of fat. In the first type of experiment the specific activity of the expired CO_2 was measured following the injection of palmitic acid-C^{14}-labeled rat chylomicrons or labeled FFA, with and without heparin. In the second type of experiment, the respiratory quotients (RQ) of carbohydrate-fed rats were determined with and without heparin injection.

Male Sprague-Dawley rats weighing between 175 and 300 g. were used. They were housed in individual cages. Twenty-four hours before an experiment they were offered only sugar tablets to eat and their drinking water was replaced with a solution of 10 per cent sucrose in 0.5 per cent NaCl. All rats subsequently used had eaten 5 to 10 g. of sugar and had drunk 50 to 125 ml. of the sucrose-saline solution. In addition, 30 minutes before an experiment each animal received 1 or 2 ml. of 50 per cent sucrose by stomach tube. All injections were given by tail vein. Experimental animals received 1 ml. of physiologic saline containing 0.25 mg. heparin; control animals received the saline only.

When the specific activity of the expired CO_2 was being measured, carbohydrate-fed rats received either heparin or saline intravenously, followed by 1 ml. of labeled chylomicrons or FFA. The chylomicrons were prepared by methods previously described (6) from chyle obtained from a cannulated rat which had previously been fed olive oil containing a trace amount of palmitic acid-1-C^{14}. Previous experience (2) has shown that 97 per cent of the chylomicron label is in the triglycerides. The preparation of FFA was made by the method previously described (3). It contained palmitic acid-1-C^{14} bound to bovine serum albumin and dissolved in Krebs-Ringer solution buffered at pH 7.2. Following injection of the label, the animal was immediately placed in a train with the

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TABLE 1. EFFECT OF HEPARIN ON THE $C^{14}O_2$ EXCRETION AND ON THE SPECIFIC ACTIVITY OF THE CO_2 AFTER THE INJECTION OF C^{14}-LABELED CHYLOMICRONS OR C^{14}-LABELED FREE FATTY ACIDS (FFA)

No. of Rats	Treatment	$C^{14}O_2$	Significance of Difference		S.A. of CO_2	Significance of Difference	
			t	p		t	p
		per cent of dose			*cpm./mmole*		
8	C^{14}-Chylos + saline	1.30 ± 1.25 *			1400 ± 1195 *		
8	C^{14}-Chylos + heparin	4.42 ± 3.62	2.15	0.05	5039 ± 3462	2.51	0.025
9	C^{14}-FFA + saline	9.44 ± 6.23			776 ± 496		
9	C^{14}-FFA + heparin	5.28 ± 3.08	1.69	0.1	506 ± 265	1.89	0.05–0.1

* Standard deviation.

following characteristics: Room air was drawn through soda lime and then through a cylindrical glass chamber just large enough to accommodate the rat comfortably. Moisture was condensed out by passing the air around a container of solid CO_2 in ethanol. The dry air was bubbled through sintered glass into 100 ml. of a solution of about 0.25 N Hyamine in methanol, as devised by Fredrickson and Ono (7). A final $Ba(OH)_2$ trap indicated that no CO_2 escaped through the Hyamine. Collections continued for 30 minutes, after which time 5 ml. aliquots were titrated in triplicate with 0.25 N HCl and 2 ml. aliquots were counted in duplicate in a liquid scintillation counter after the addition of 15 ml. of a 0.5 per cent solution of diphenyloxazole in toluene.

When the respiratory quotient was being measured, the animal was placed in a closed-circuit train through which CO_2-free room air was circulated. A pump circulated the air through the animal chamber, a CO_2 absorbant (100 ml. of 0.5 N KOH), a spirometer of 6 l. volume, and the pump. The animal remained in the train for 1 hour following injection. Oxygen consumption was read directly from the spirometer scale with a maximum error of 3 per cent. Excreted CO_2 was measured by titration of the KOH with 0.5 N HCl in triplicate samples. Individual titrations were within 2 per cent of the mean. The experiments were performed in an air-conditioned room and it was assumed that during the collection temperature and atmospheric pressure did not vary significantly.

It has been shown by Grossman *et al.* (8) that the intravenous injection of heparin in fasting rats is followed by an increase in the FFA content of the blood. It was necessary, however, to demonstrate the same phenomenon in carbohydrate-fed animals. Sixteen such rats were divided into two groups, one of which received heparin in saline and the other saline only.

Fifteen minutes later each animal was anesthetized with intravenous pentobarbital and 5 ml. of blood was rapidly withdrawn from the aorta into a syringe containing a small amount of dry heparin. The blood was immediately transferred to centrifuge tubes in a bath of ice water. The blood was centrifuged at 0°C and 1 ml. of plasma extracted in duplicate. The FFA were determined by the method of Dole (9). We had previously determined, as had Grossman *et al.* (8), that incubation of rat blood in ice water completely inhibited the activity of lipoprotein lipase. It was necessary to do this to prove an *in vivo* effect of heparin on FFA levels.

RESULTS

The results of the experiments in which labeled chylomicrons or FFA were injected with and without heparin are presented in Table 1. Following the injection of chylomicrons, heparin more than tripled the amount of $C^{14}O_2$ expired, as well as tripling the specific activity of the CO_2. Following the injection of labeled FFA, there was an actual decrease both in the amount of $C^{14}O_2$ and in the specific activity of the CO_2. Although not statistically significant ($p = 0.05$-0.1), this probably represents a dilution of the circulating isotope caused by the elevation in plasma FFA brought about by heparin, as is shown in Table 2.

In addition to showing a significant elevation in plasma FFA levels following the injection of heparin, Table 2 also shows that heparin lowers the respiratory quotient. In one experiment, 11 rats that received saline are compared with 10 rats that received he-

TABLE 2. THE SERUM FREE FATTY ACID (FFA) LEVELS AND THE RESPIRATORY QUOTIENTS OF CARBOHYDRATE-FED RATS INJECTED WITH SALINE OR HEPARIN

No. of Rats	Treatment	FFA	RQ	Significance of Difference	
				t	p
		meq./l.			
8	Saline	0.17 ± 0.033 *			
8	Heparin	0.24 ± 0.048		3.03	<0.01
11	Saline		0.97 ± 0.070 *		
10	Heparin		0.87 ± 0.065	3.22	<0.01
10 †	Saline		1.02 ± 0.08		
10	Heparin		0.90 ± 0.09	4.20	<0.005

* Standard deviation.
† Saline and heparin injected into same animals.

parin. In a second experiment, 10 rats served as their own controls; the RQ was determined first after saline and then after heparin. In the interval between these measurements, additional carbohydrate was given by stomach tube. In both experiments the RQ of the animals receiving saline approached unity, indicating that their energy requirements were being met primarily through the oxidation of carbohydrate. In both experiments there was a highly significant decrease in the RQ after the injection of heparin.

DISCUSSION

As pointed out by French and Morris (5), who demonstrated an increased rate of excretion of $C^{14}O_2$ in rats receiving labeled chyle and heparin as compared to saline controls, the effect is probably explained by the observation that FFA is more readily available for oxidation than is triglyceride. The injection of heparin with chylomicrons, through the release of lipoprotein lipase, permitted a more rapid conversion of the triglyceride to FFA. This interpretation is strengthened by the observation that when the label was injected as FFA, heparin had no such effect. It should not be inferred from these findings that retransport of chylomicron triglyceride as plasma FFA is an obligatory step preceding oxidation. That some chylomicrons may be removed from the blood and oxidized without recycling as plasma FFA has been emphasized by Fredrickson *et al.* (10).

Of more interest is the observation that heparin will lower the RQ of carbohydrate-fed rats. The injection of heparin apparently results in an obligatory oxidation of fat, even in the presence of adequate amounts of carbohydrate. One can only speculate on the mechanism involved here, but it seems not unlikely that the release of lipoprotein lipase causes the hydrolysis of adipose or other tissue triglyceride, and that the accumulation of FFA, as reflected in an elevation of plasma FFA, brings about an obligatory oxidation.

REFERENCES

1. Korn, E. D., and T. W. Quigley, Jr. *J. Biol. Chem.* **226:** 833, 1957.
2. Havel, R. J., and D. S. Fredrickson. *J. Clin. Invest.* **35:** 1025, 1956.
3. Bragdon, J. H., and R. S. Gordon, Jr. *J. Clin. Invest.* **37:** 574, 1958.
4. French, J. E., and B. Morris. *J. Physiol.* **138:** 326, 1957.
5. French, J. E., and B. Morris. *J. Physiol.* **140:** 262, 1958.
6. Bragdon, J. H. *J. Lab. Clin. Med.* **52:** 564, 1958.
7. Fredrickson, D. S., and K. Ono. *J. Lab. Clin. Med.* **51:** 147, 1958.
8. Grossman, M. I., L. Palm, G. H. Becker, and H. C. Moeller. *Proc. Soc. Exptl. Biol. Med.* **87:** 312, 1954.
9. Dole, V. P. *J. Clin. Invest.* **35:** 150, 1956.
10. Fredrickson, D. S., D. L. McCollester and K. Ono. *J. Clin. Invest.* **37:** 1333, 1958.

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The fatty acid composition of chylomicrons of chyle and serum following the ingestion of different oils

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SUMMARY

Chylomicrons were isolated from the thoracic duct chyle and from the blood serum of rats, and from the serum of human subjects following the ingestion of different natural oils. The fatty acid composition of the chylomicrons was compared with that of the dietary fat by gas chromatography and was found to be very similar.

Dole *et al.* (1) have recently reported a remarkable constancy of fatty acid composition of chylomicrons separated from human plasma during the period of absorption of fats differing greatly in fatty acid composition. They speculated as to possible mechanisms involved. Either the fatty acids were chemically altered during absorption through the intestinal wall or there was rapid recycling between chylomicron fatty acids and tissue fatty acids. The purposes of this paper are to report the analyses of chyle and serum chylomicrons following the feeding of different fats to rats, and to attempt to confirm Dole's findings in human blood chylomicrons.

METHODS

Polyethylene cannulae were inserted in the thoracic ducts of three rats. The following day the animals were fed 1 ml. of coconut oil, olive oil, and cod liver oil, respectively. The chyle was collected and the chylomicrons isolated and washed as previously described (2). In brief, the chyle was layered under 0.15 M NaCl and centrifuged at 100,000 $\times$ g for 30 minutes. The butterlike material at the top of the tube was re-emulsified in 0.15 M NaCl and recentrifuged. This process of washing was repeated. In order to get rat serum chylomicrons, groups of four fasting rats were fed each of the three oils. They were bled from the aorta 3 hours later; the blood from each group was pooled, and the serum was treated essentially as the chyle.

Two young women, who had spent several months in the Clinical Center as volunteer controls and who had been on a mixed diet (limited to 35 g. of fat per day) were fed a formula breakfast in which the sole source of fat was either coconut oil or corn oil. The dose was 2 g. per kg. of body weight. They were bled fasting and 6 hours postprandially. Their serum samples were treated differently from the rat serum samples. Layering and washing took place in saline containing 0.01 M phosphate buffer at pH 7.4 and 0.05 per cent of the disodium salt of ethylene diamine tetraacetic acid. Centrifugation was carried out for 30 minutes at only 26,000 $\times$ g and the chylomicrons were subjected to three washings in addition to the original separation.

Samples of the fats fed the rats, of the formula diets fed the human subjects, and of the chylomicrons were extracted in chloroform-methanol as previously described (2). Aliquots of the chloroform phase were evaporated to dryness at 20°C under nitrogen. Methyl esters were prepared by the transesterification procedure of Stoffel *et al.* (3), which involves refluxing the lipids with methanol, through which dry HCl gas has been passed until the HCl concentration is 5 per cent (w/w) in the methanol. The methyl esters were then extracted into redistilled hexane, and concentrated by evaporation under nitrogen. Aliquots were analyzed by gas chromatography.

The column used was a glass column, 8 feet long, 5 mm. I.D., filled with chromosorb W, siliconized with dimethyl dichlorosilane according to the method of Horning *et al.* (4), on which 15 per cent (w/w) of a polyester of adipate diethylene glycol had been coated. Analyses were performed at 200°C with a gas flow of 100 ml. per minute. Detection devices used in this study included a hot wire katharometer (model K5,

TABLE 1. THE PERCENTAGE FATTY ACID COMPOSITION OF 3 OILS COMPARED WITH THE COMPOSITION OF CHYLOMICRONS SEPARATED FROM CHYLE AND SERUM OF RATS FOLLOWING INGESTION OF THE OILS

Fatty acids *	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:0 †	20:1 †	22:1 †
Olive oil		0.0	16	1.8	2.1	67	13			
Chyle chylomicrons		0.0	16	2.2	2.9	63	15			
Serum chylomicrons		0.0	17	2.3	2.0	64	15			
Cod liver oil		6.2	18	16	3.3	32	1.1	2.0	10	12
Chyle chylomicrons		6.5	21	17	4.1	34	4.6	1.0	8.1	4.5
Serum chylomicrons		5.1	21	15	4.6	33	6.3	1.5	6.7	6.7
Coconut oil ‡	71	17	5.7	0.0	1.9	3.2	1.0			
Chyle chylomicrons	56	20	8.2	0.0	2.1	8.2	4.3			
Serum chylomicrons	37	16	17	1.2	3.9	16	8.6			

* The first number refers to the number of carbon atoms; the second number refers to the number of double bonds.

† The fatty acids labeled 20:0, 20:1, and 22:1 are designated as such from retention volume data on adipate-diethylene glycol polyester gas chromatography columns. Their identity has not been confirmed by chemical determinations or by the use of other columns. They may represent more highly unsaturated esters of shorter chain length.

‡ The acids shorter than 12:0 have been ignored in computing the composition.

Burell and Co.) and an argon-ionization detector similar to that developed by Lovelock (5), employing a 300 μc. radium foil. Voltage and amplifier settings were kept constant throughout the analyses.

At least two analyses of each sample were performed. The volume of the aliquots analyzed was varied in order to present long- and short-chain esters of the mixture to the detector cells in approximately equal concentrations, thus permitting more accurate quantification. Acids present in trace amounts have been ignored.

RESULTS

The fatty acid composition of the three fats that were fed to the rats and of the chylomicrons obtained both from the chyle and from the serum are presented in Table 1. In the case of olive oil and cod liver oil feeding, the composition of the chylomicrons, whether from chyle or serum, exhibits a remarkable similarity to the dietary fat. The only significant difference in this regard occurs in the chylomicrons after cod liver oil feeding. This oil contains very little linoleic (18 : 2) acid, and it appears that some of this acid was added from nondietary sources in the intestinal mucosa. In the rat fed coconut oil, which has a very high content of lauric (12 : 0) acid, there is a significant decrease in the concentration of this acid in the chyle chylomicrons. This is not surprising in view of the findings of Bloom *et al.* (6) that only 15 to 55 per cent of fed lauric acid is absorbed via the lymph in rats; the remainder of this relatively short-chained acid is presumably absorbed via the portal system, as less than 5 per cent of the dose could be recovered in feces and intestinal contents. Shorter acids are absorbed entirely through the portal system and for this reason have been ignored in computing the composition of the

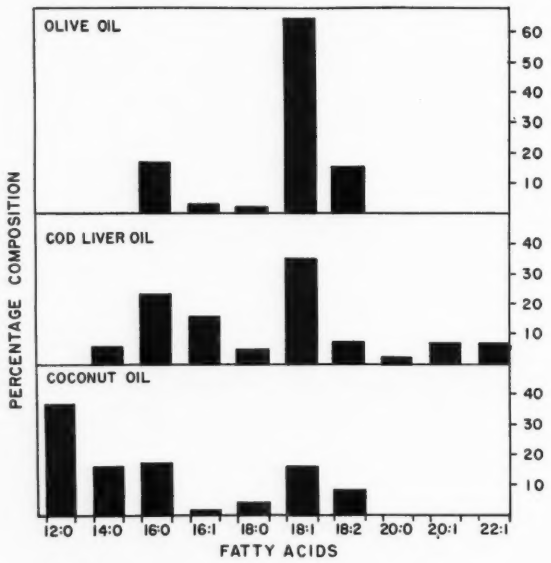

FIG. 1. The percentage fatty acid composition of rat serum chylomicrons following the ingestion of 3 different oils. The fatty acids are identified as follows: the first figure refers to the number of carbon atoms; the second figure refers to the number of double bonds.

TABLE 2. THE PERCENTAGE FATTY ACID COMPOSITION OF 2 OILS COMPARED WITH THE COMPOSITION OF HUMAN SERUM CHYLOMICRONS 6 HOURS FOLLOWING INGESTION OF THE OILS

Fatty acids	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Corn oil	0.0	0.5	17	0.0	1.2	25	57
Chylomicrons	0.7	0.7	16	1.2	2.4	28	51
Coconut oil *	73	19	6	0.0	2.0	0.0	0.0
Chylomicrons	24	23	27	1.2	5.0	16	3.2

* The source of this oil differed from that fed the rats.

coconut oil. Comparing the composition of the chyle chylomicrons with the composition of the coconut oil, it is apparent that oleic (18 : 1) and linoleic (18 : 2) acids have been added from nondietary sources. This is the only case in which the serum chylomicrons differ significantly from the chyle chylomicrons. There is a further reduction in the short-chain acids with a relative increase in those of 16 and 18 carbon atoms. The chemical composition of the serum chylomicrons is shown graphically in Figure 1.

In the two human subjects one can compare only the serum chylomicrons with the dietary fat, and these data are presented in Table 2. When corn oil, which contains long-chain acids exclusively, was fed, the fatty acid composition of the serum chylomicrons was almost identical to that of the corn oil. When coconut oil, with a preponderance of short-chain acids, was fed, the result was similar to that observed in the rat, and presumably for the same reason: that most of the lauric acid (12 : 0) was absorbed via the portal system. The composition of the serum chylomicrons is shown graphically in Figure 2.

DISCUSSION

Dole *et al.* (1) have reviewed the older literature, with its conflicting data, on the effect of dietary fat on the composition of chylomicrons. In most cases whole lymph, rather than chylomicrons only, was analyzed.

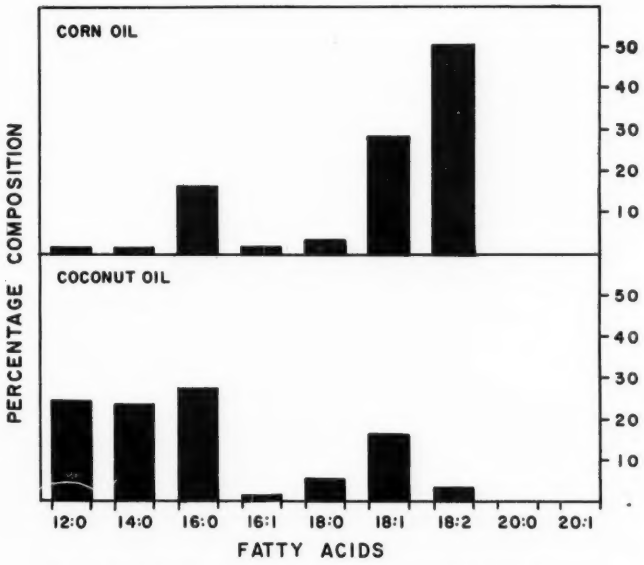

FIG. 2. The percentage fatty acid composition of human serum chylomicrons following the ingestion of 2 different oils. The fatty acids are identified as follows: the first figure refers to the number of carbon atoms; the second figure refers to the number of double bonds.

It is well known that whole lymph contains lipids other than those immediately absorbed from the intestine. Dole and his co-workers, although they did find significant differences in the fatty acid composition of serum chylomicrons following the feeding of coconut oil or corn oil, stressed their relative similarity and emphasized the differences between the composition of the chylomicrons and the composition of the dietary fat. The findings presented here differ from those of Dole, and show a similarity in composition between chylomicrons and the dietary fat. This discrepancy can perhaps be explained by the difference in methods used for the separation of the chylomicrons. Dole and his co-workers layered human serum under physiological saline and centrifuged at 100,000 $\times$ g for 30 minutes. They then analyzed the material in the top of the tube. This gravitational field is sufficient to cause many low density lipoproteins, as well as chylomicrons, to rise to the top of the tube. Furthermore, they did not wash their chylomicron preparations, which were presumably contaminated with fatty acids not of immediate dietary origin. The human chylomicrons analyzed here, on the other hand, were removed from the top of the tube after centrifuging for 30 minutes at only 26,000 $\times$ g and they were washed three times to free them of contaminating lipoproteins. It is true that the rat chylomicrons described here were isolated under centrifugal conditions identical to those of Dole, but rat serum contains very low concentrations of low density lipoproteins, only a small fraction of those often encountered in human serum, and again the chylomicrons were washed.

Ten ml. of the fasting human serum yielded so few chylomicrons that analysis was impossible. The usual sample of fatty acid esters placed on the column was in the neighborhood of 30 μg.

In the rat fed cod liver oil, linoleic acid appeared to have been added to the chyle chylomicrons from nondietary sources. In the coconut oil-fed rat both oleic and linoleic appear to have been added. It is possible that bile, pancreatic juice, and digested epithelial cells are the source of these acids.

The only significant change in the composition of chylomicrons occurring after they have entered the blood stream appears in the case of coconut oil-fed rats, in which lauric (12 : 0) and myristic (14 : 0) acids appear to have been selectively removed, with the result that the long-chain acids appear relatively more abundant. No explanation is offered for this observation.

The fact that the fatty acid composition of serum chylomicrons closely resembles that of the chyle chylomicrons indicates that very little, if any, exchange or recycling occurs between chylomicron fatty acids and tissue fatty acids. At least this appears true for the longer-chained acids, a fact which tends to validate recent work from this laboratory on the metabolism of chylomicrons (7, 8). Such is not true for the free fatty acids of the blood, which, as shown by Dole *et al.* (1), can be only slightly influenced in composition by short-term changes in the diet, and which presumably rapidly reach chemical equilibrium with adipose tissue (9).

REFERENCES

1. Dole, V. P., A. T. James, J. P. W. Webb, M. A. Rizack, and M. F. Sturman. *J. Clin. Invest.* **38:** 1544, 1959.
2. Bragdon, J. H. *J. Lab. Clin. Med.* **52:** 564, 1958.
3. Stoffel, W., F. Chu and E. H. Ahrens, Jr. *Anal. Chem.* **31:** 307, 1959.
4. Horning, E. C., E. A. Moscatelli and C. C. Sweeley. *Chem. & Ind. London,* 751 (1959).
5. Lovelock, J. E. *J. Chromatog.* **1:** 35, 1958.
6. Bloom, B., I. L. Chaikoff and W. O. Reinhardt. *Am. J. Physiol.* **166:** 4451, 1951.
7. Bragdon, J. H., and R. S. Gordon, Jr. *J. Clin. Invest.* **37:** 574, 1958.
8. Fredrickson, D. S., D. L. McCollester and K. Ono. *J. Clin. Invest.* **37:** 1333, 1958.
9. Fredrickson, D. S., and R. S. Gordon, Jr. *Physiol. Revs.* **38:** 585, 1958.

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Effect of cerebroside preparations on sterol excretion in the rat*

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SUMMARY

The addition of cerebroside preparations to synthetic diets fed to rats caused an increased excretion of sterol in the feces as measured by the Liebermann-Burchard reaction. The mixed fatty acid esters obtained by hydrolyzing the cerebrosides had a similar but less marked effect. Both cerebrosides and fatty acid esters increased the concentration of Liebermann-Burchard-positive sterol in the feces. The amounts of sterol in the diet were insufficient to contribute significantly to the fecal sterol and no component of the cerebroside preparations other than fatty acids was found to affect sterol excretion. The activity displayed by the fatty acid fraction did not seem to be restricted to one particular fatty acid. Cholesterol was found to be the main chromogenic sterol in the feces during the first week that cerebrosides were fed. Possible mechanisms for this response to the feeding of cerebrosides are discussed.

The feeding of long-chain mono-unsaturated fatty acids such as erucic acid (C_{22}) and nervonic acid (C_{24}) to rats has been shown to increase the concentration of cholesterol in the adrenals and liver and to promote the excretion of fecal cholesterol in the absence of dietary sterol. Saturated fatty acids with chain length of C_{16} or greater were also found to increase the fecal excretion of sterol but seemed to have little or no effect on tissue levels (1). Nervonic acid is a component of the cerebrosides found in the central nervous system, and the effect of crude preparations of these compounds on cholesterol metabolism was also investigated. Preliminary results showed that they increased fecal sterol excretion but had little effect on tissue cholesterol levels (1). Jones and his associates have reported that the feeding of cerebrosides causes an increased excretion of fecal sterol (2, 3) and further, they have found that administration of cerebroside preparations lowers the blood cholesterol level of patients and of experimental animals with hypercholesterolemia (4, 5). These preparations also appear to increase the turnover rate of serum cholesterol (6).

In the present study an attempt has been made to separate and identify the cerebroside fractions which affect the excretion of fecal sterol in rats and to examine further the possible relationship between this effect and that observed with individual fatty acids.

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METHODS

Three different cerebroside preparations were used in these experiments. Beef brain or spinal cord was obtained in the frozen state and was extracted in 50 lb. lots by the method of Carter *et al.* (7). A commercial preparation of cerebrosides made from a mixture of brain and spinal cord[1] was used for most of the later work. The galactose, phosphorus, and sterol content of these different preparations is shown in Table 1.

The galactose content was determined by a direct anthrone method as follows:[2] Anthrone reagent was prepared by dissolving 500 mg. of anthrone in 200 ml. of concentrated sulfuric acid and diluting the solution to 500 ml. with 54 per cent sulfuric acid (150 ml. of concentrated sulfuric acid diluted with 210 ml. of water). This reagent was stored in a dark bottle and could be used for about 2 weeks. Cerebroside preparations were dissolved in ethyl alcohol with warming and diluted to a concentration of about 1 mg. per ml. At

[1] The author is indebted to Dr. David Klein, of the Wilson Laboratories, Chicago, for generous supplies of cerebroside preparation WT-5.

[2] Dr. J. F. Richards collaborated in the development of this method.

this concentration the cerebrosides tend to precipitate slowly on standing at room temperature, so the solutions were normally made up on the day on which they were analyzed. In some cases (e.g., psychosine sulfate) it was necessary to use 40 : 60 ethanol-water to dissolve the sample and in other cases the sample was dissolved in a small amount of chloroform and then diluted with ethanol to the required concentration. It was found that 4 per cent chloroform in the ethanol solution did not interfere with the determination.

One-half ml. of each cerebroside solution was transferred to a test tube (25 × 150 mm.) and 10 ml. of anthrone reagent was added with mixing. When the anthrone is made up in diluted sulfuric acid, it is not necessary to cool the tube during this addition (8). The tubes were placed in a boiling water bath for exactly 8 minutes, then removed and placed immediately in cold water. After cooling, they were read in an Evelyn colorimeter with a 620 mμ filter against a blank which was prepared with 0.5 ml. of ethanol and 10 ml. of anthrone reagent and carried through the same heating procedure. A standard curve was prepared by using 0.5 ml. samples of aqueous galactose solutions containing from 20 to 400 μg. per ml. of galactose.

Phosphorus was determined by the method of King (9) after oxidation of the samples with 70 per cent perchloric acid. For measurement of sterol content, 400 mg. of cerebroside were taken up in 10 ml. of hot acetone-alcohol (1 : 1), the solution was allowed to cool, filtered, and the sterol in a 5 ml. aliquot was precipitated with digitonin and measured by the Sperry-Webb procedure (10).

Recrystallization of Wilson Cerebrosides. Two hundred g. of crude cerebrosides were dissolved in 1000 ml. of chloroform-methanol (3 : 1) and precipitates were removed after cooling to room temperature, to 3°C and to —16°C (Fractions 1 to 3). The filtrate was then mixed with an equal volume of acetone and precipitates were removed at room temperature and —16°C (Fractions 4 and 5). The amounts of these various fractions and their sugar and phosphorus content are listed in Table 1.

Preparation of Partial Hydrolysis Products of Cerebrosides. The method of Carter and Fujino (11) was used for the preparation of psychosine sulfate from 80 g. lots of Wilson cerebrosides. In preparations 1 and 2 (Table 1), the cerebrosides were first recrystallized from glacial acetic acid and chloroform-methanol (3 : 1), respectively, while in preparation 3 the cerebrosides were used without purification. The purified cerebrosides gave somewhat better yields of psychosine sulfate, but when the losses on recrystallization were taken into account, the best over-all yield was obtained by using the crude cerebrosides directly. Since the galactose content of the latter preparation appeared to be satisfactory, no recrystallization was carried out in preparing material for the feeding tests.

TABLE 1. ANALYSIS OF CEREBROSIDE PREPARATIONS

Cerebroside Preparation	Yield	Galactose *	Phosphorus	Sterol †
	per cent	*per cent*	*per cent*	*per cent*
Beef brain		16.5	1.26	0.02
Beef spinal cord		16.4	1.20	0.13
Wilson Laboratories				
Lots 1-3		15.4	1.42	0.02
Lots 4-5		11.6	1.91	0.05
Recrystallized				
Fraction 1	10	26.6	0.22	
Fraction 2	18	19.0	0.72	
Fraction 3	42	14.3	1.35	0.01
Fraction 4	8	10.8	2.09	
Fraction 5	16	6.8	2.62	
Residue	2	1.2	2.53	
Psychosine sulfate				
1	15	32.1		
2	11	30.8		
3	20	30.8		
Psychosine	16	36.5	0.05	
Ceramide	36	3.2	0.25	0.03

* Theoretical values for galactose content are as follows: cerebrosides (22% approx.), psychosine sulfate (35.3%), and psychosine (39.0%). Pure ceramide contains no galactose. The high value for Fraction 1 of the recrystallized cerebrosides may be due to the presence of gangliosides which contain 40 to 43% galactose.

† Digitonin-precipitable, Liebermann-Burchard-positive sterol calculated as cholesterol.

For regenerating the free base, the product from three 80 g. runs was dissolved in 400 ml. of water and 2.5 per cent barium hydroxide solution was added until precipitation was complete at a pH of 11 to 12. The mixture was then warmed on a steam bath until the precipitate coagulated. Some difficulty was encountered in filtering this precipitate, so most of the supernatant was decanted and the residue was centrifuged. After several washings with water in the centrifuge tube, the material filtered readily; it was washed with water until neutral, dried, and then extracted with three 1-l. portions of hot alcohol. The combined extracts were allowed to cool to room temperature and filtered. The alcohol was removed from the filtrate to yield 40 g. of a pale yellow product, which was probably a mixture of psychosine and dihydropsychosine.

Ceramide was prepared from 20 g. lots of Wilson cerebrosides by hydrolysis with a mixture of acetic and sulfuric acids according to the procedure of Klenk (12). The crude residue from the ether extract was used without further purification for the feeding tests. Analytical figures for both psychosine and ceramide preparations are given in Table 1.

Preparation of Mixed Fatty Acid Esters from Cerebrosides. To a solution of 75 ml. of concentrated sulfuric acid in 1500 ml. of methanol, 150 g. of cerebrosides were added, and the mixture was refluxed for 6 hours (13). After cooling, it was extracted with 3 portions of 1000 ml. of petroleum ether (b.p. 35°-60°C). The combined extract was washed twice with 400 ml. portions of 50 per cent methanol in water and once with 300 ml. of water. It was then dried with sodium sulfate and the petroleum ether was removed *in vacuo*. This yielded 65 to 70 g. of fatty acids in the form of methyl esters. The preparation from beef spinal cord cerebrosides was a white solid which melted on warming. That from beef brain cerebrosides was a light brown and that from Wilson cerebrosides a dark brown solid. However, the latter could be distilled *in vacuo* to give a product which was only slightly colored.

Fractional Crystallization of Mixed Fatty Acid Esters from Wilson Cerebrosides. Two hundred thirty g. of distilled methyl esters (iodine value 26.2, saponification equivalent 341) was recrystallized twice from 4600 ml. of methanol at 3°C to give 84 g. of precipitate (iodine value 3.6, saponification equivalent 371). The first filtrate was concentrated to 850 ml. and cooled to 3°C, giving 46 g. of precipitate (iodine value 29.3). This was recrystallized from 850 ml. of methanol at 3°C and the filtrates from these two crystallizations were combined, concentrated to 400 ml. and cooled to 3°C to remove a further precipitate. The esters remaining in solution weighed 60 g. (iodine value 54.3, saponification equivalent 292).

Fractional Distillation of Mixed Fatty Acid Esters from Wilson Cerebrosides. Distilled methyl esters (310 g.) were redistilled at reduced pressure through a Todd fractionating column.[3] The results are shown in Table 2.

Preparation of Mixed Bases from Cerebrosides. Cerebrosides prepared from beef brain or spinal cord were hydrolyzed with sulfuric acid in methanol and the fatty acid esters were extracted as described previously. The ether-soluble bases were then recovered from the methanol layer by the extraction procedure described by Carter *et al.* (13). The ether extract from 300 g. of hydrolyzed spinal cord cerebrosides was washed, dried with sodium sulfate, concentrated to 750 ml., and allowed to stand at 3°C. This gave 12 g. of white precipitate (melting range 70°-73°C). Cooling the extract to —16°C gave a further 30 g. of light yellow precipitate (melting range 60°-65°C). The residue from the ether was a brown gummy material (15 g.), from which it was difficult to remove the last of the solvent. Hydrolysis of beef brain cerebrosides gave smaller quantities of product (2.5 g. at 3°C and 15 g. at −16°C), which were more colored than those from spinal cord but melted in the same temperature range. The sulfates and triacetyl derivatives of the mixed bases from beef spinal cord cerebrosides were prepared by the methods of Carter *et al.* (13). The triacetyl derivative melted at 99°-100°C. Carter's triacetylsphingosine melted at 101°-102°C and triacetyldihydrosphingosine melted at 102°-103°C.

TABLE 2. FRACTIONAL DISTILLATION OF FATTY ACID METHYL ESTERS FROM WILSON CEREBROSIDES

Fraction	Weight	Boiling Range	Iodine Value	Saponification Equivalent
	g.	*°C*		
1	38	155–63	14	284
2	76	167–72	19	300
3	18	180–87	30	301
4	17	197–205	47	348
5	82	215–25	36	384
6	51	230–33	31	399
Residue	14	——	31	399

Dietary Experiments. Feeding experiments were conducted in the same way as those described previously (1). Male rats of the Sprague-Dawley strain, weighing 80 to 90 g., were caged in groups of three. Their feces were discarded for the first 2 days of the feeding period, following which they were collected and the food intake measured for two successive 7-day periods. The diets had the following composition, calculated on a per cent by weight basis: casein, 19 to 23 per cent; lipid preparation, 0 to 15 per cent; salt mixture, 4 to 5 per cent; Cellu flour, 5 per cent; and glucose, to make 100 per cent. The casein was increased in the fat-containing diets in an attempt to keep constant the proportion of calories derived from protein. An adequate supplement of water-soluble vitamins was added to each diet, but fat soluble vitamins were omitted since most of the experiments were of short duration and it was felt that deficiencies were unlikely to occur. The sources of the dietary materials

[3] Todd Scientific Co., Springfield, Pa.

and the composition of the vitamin mixture have been described previously (14).

Determination of Fecal Sterols. In most experiments the fecal sterol was measured colorimetrically by the Sperry-Webb procedure (10). Weighed aliquots of about 100 mg. of dried, powdered feces were extracted 3 times with 3 ml. portions of boiling acetone: alcohol (1 : 1), the combined extracts were made to 10 ml., filtered, and a 2 to 3 ml. aliquot used for the determination. The results of the colorimetric assay were calculated as though the chromogenic sterol were cholesterol. However, some fecal sterols develop colors at different rates and intensities (15). Therefore these results do not necessarily give a true picture of the total excretion of fecal sterols.

In earlier experiments in which erucic acid was fed to rats, the fecal sterols were separated by chromatography on alumina, and it was shown that cholesterol was the main chromogenic sterol excreted under those conditions (1). An attempt to carry out a similar separation of the fecal sterols of rats fed cerebrosides was complicated by the large amounts of unabsorbed cerebrosides in the feces, which made it difficult to obtain quantitative recovery of sterols in a form suitable for chromatography. However, the following procedure has been found satisfactory for the purpose: Aliquots of feces weighing 10 g. were extracted with ether for 48 hours in a Soxhlet apparatus. The ether extract was centrifuged, the precipitate washed with small amounts of ether, and the washings were added to the original supernatant. The ether solution was then concentrated to 25 ml. and 5 volumes of acetone were added. The precipitate was centrifuged and washed with acetone. The supernatant and washings were combined, taken to dryness, and the residue was taken up in 10 to 20 ml. of ether. Four volumes of acetone were added, the solution was cooled to 0°C, and a further precipitate was separated. The supernatant and washings were combined and taken to dryness as before, giving a residue which weighed approximately 1 g. This was taken up in a small volume of Skellysolve B and added to a column (8.0 cm. in length by 2.9 cm. in diameter) containing 30 g. of Florisil.[4] The column was eluted with a mixture of Skellysolve B and ether[5] (9 : 1), according to Radin and Kishimoto (16). A nearly quantitative recovery of chromogenic sterol was obtained in the first 400 ml. of effluent from the column with the cholesterol fraction appearing between 200 and 400 ml. The sterols eluted from the Florisil column were then rechromatographed on alumina as described in our earlier work (1). Cholesterol was identified by its position on the chromatograms and its infrared spectrum. Further, the Liebermann-Burchard color developed by fractions in the cholesterol region of the alumina chromatogram corresponded closely with that expected from the weight of the fractions if all of the material were cholesterol.

[4] An activated magnesium silicate, 60/100 mesh, obtained from the Floridin Co., Tallahassee, Fla.

[5] Skellysolve B is a mixture of hydrocarbons (mainly n-hexane) boiling at 60°-71°C. It was dried over sodium and distilled and the portion boiling at 68°-70°C was used.

RESULTS

The effects of dietary cerebrosides and cerebroside fatty acid esters on the excretion of chromogenic sterol in the feces are presented graphically in Figure 1 and results obtained by feeding erucic acid are included for comparison. These results show that unhydrolyzed cerebrosides cause a greater increase in total fecal chromogenic sterol than do the fatty acid esters derived from them. Part of this difference can be accounted for by the greater bulk of feces excreted when diets containing cerebrosides are fed, but in most experiments cerebrosides seemed also to increase the concentration of chromogenic sterol in the feces more than did the fatty acid esters (Fig. 1 and Table 3). The results obtained with erucic acid, fed at levels of 10 and 15 g. per 100 g. of diet, were comparable to those of the mixed fatty acid esters of cerebrosides, but erucic acid had little effect on sterol excretion when fed at the 5 per cent level while both cerebrosides and their fatty acid esters gave measurable effects at the 2 per cent level in the diet.

Attempts to fractionate crude cerebroside preparations by recrystallization from chloroform-methanol failed to localize the sterol excretion effect to any particular fraction, although it did appear that the fractions having the highest and lowest sugar content were somewhat less active than the others. Experiments on fractionation of the fatty acid esters either by recrystallization or by fractional distillation likewise failed to produce evidence for a single active component (Table 3).

A mixture of ceramides obtained by partial hydrolysis of a crude cerebroside preparation gave results comparable to that of the mixed fatty acid esters fed at the same level. Attempts to assess the effect of preparations of psychosine or sphingosine were complicated by the fact that such preparations were toxic even when fed at the 1 per cent level in the diet.[6] Triacetylsphingosine appeared to have no effect on fecal sterol excretion.

[6] K. K. Carroll, unpublished observations.

Effect of Cerebrosides on Absorption of Exogenous Cholesterol. A balance experiment was carried out in which a diet containing 5 per cent of Wilson cerebrosides was fed with and without added cholesterol. The results given in Table 4 indicate that 30 to 40 per cent of the exogenous cholesterol was absorbed under these conditions.

Nature of the Fecal Sterol. The fecal sterol was nearly all in the free form when diets containing unhydrolyzed cerebrosides or ceramides were fed. When 10 or 15 per cent fatty acid esters derived from cerebrosides were fed, most of the fecal sterol was esterified, but when they were fed at lower levels, most of the sterol was in the free form. In one experiment feces were collected for prolonged periods and the chromogenic sterol was determined on a series of weekly collections (Fig. 2). Rats fed a diet containing 15 per cent of Wilson cerebrosides excreted large amounts of chromogenic sterol during the first two collection periods. The amount then dropped abruptly to about one-half the initial value and the new level was maintained for at least 5 weeks. When fatty acid esters derived from cerebrosides were fed, no change in the rate of excretion of chromogenic sterol was observed at the end of 2 weeks, but a decrease was observed after 5 to 6 weeks. The rats grew better on the cerebroside diet than on the diet containing cerebroside fatty esters, and they ate on the average 19 g. per day of the cerebroside diet, compared to 15 g. per day of the diet containing fatty acid esters. It should be noted that the animals were given no fat-soluble vitamins and this probably influenced their rate of growth (14).

The fecal sterols from rats fed the diet containing cerebrosides, obtained during the first and third collection periods, were extracted as described before and chromatographed first on Florisil and then on alumina. Cholesterol appeared to be the main chromogenic sterol excreted during the first collection period but a different chromatographic pattern was observed for fecal sterols of the third collection period. Further experiments on this aspect of the problem are in progress.

DISCUSSION

The present study has given no indication that any one specific component of crude cerebroside preparations is responsible for their effect of increasing fecal sterol excretion in the rat. Recrystallization of Wilson

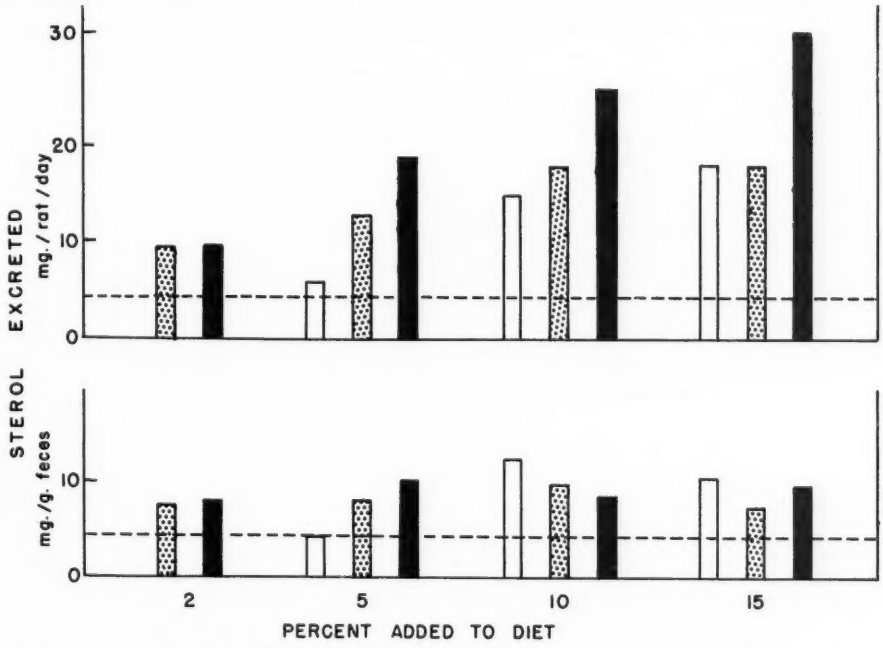

FIG. 1. Amounts of digitonin-precipitable, Liebermann-Burchard-positive fecal sterols in rats fed synthetic diets containing different amounts of cerebrosides, cerebroside fatty acid esters, or erucic acid. Solid bars refer to cerebroside, stippled bars to cerebroside fatty acid ester, and open bars to erucic acid experiments. The upper figure gives total fecal sterol; the lower part gives the concentration of fecal sterol. Dotted lines indicate amounts of fecal sterol in rats on a fat-free synthetic diet. Values given represent averages of experiments carried out over a period of several years. Each diet was fed to one group of three rats, but in most cases the experiment was repeated at least once.

cerebrosides yielded a number of fractions which were equally effective in increasing the fecal sterol output, although their sugar content ranged from 19 to 7 per cent and their phosphorus content varied from 0.7 to 2.6 per cent. Fractionation of the mixed fatty acid esters obtained by hydrolyzing Wilson cerebrosides also failed to produce evidence for a single active component. It therefore appears that this effect of cerebroside preparations is not due solely to their content of any individual fatty acid such as nervonic acid. Unhydrolyzed cerebrosides caused a greater increase in the total fecal sterol output than did the mixture of fatty acid esters obtained on hydrolysis, but both cerebrosides and fatty acid esters increased the concentration of chromogenic sterol in the feces (Fig. 1).

No evidence was obtained to indicate that components of cerebroside preparations other than fatty acids affect sterol excretion. Sphingosine and psychosine could not be assessed satisfactorily because of their toxicity, but triacetylsphingosine appeared to be inactive. The small amounts of sterol present in the cerebroside preparations were insufficient to contribute significantly to the fecal sterol.

It is of interest to consider the mechanisms by which cerebroside preparations may affect the fecal excretion of endogenous sterol. One possibility is that they interfere with the reabsorption of sterols from the intestine. In order to test this, a balance experiment was carried out to determine the effect of cerebrosides on the absorption of cholesterol added to the diet, and it was found that 30 to 40 per cent of the cholesterol was absorbed from a diet containing 0.25 per cent cholesterol and 5 per cent of Wilson cerebrosides (Table 4). This is comparable to values obtained by Lin *et al.* for absorption of cholesterol from a fat-free diet or a diet containing tripalmitin (17), but is significantly lower than those observed when cholesterol was fed with diets containing unsaturated fats or fatty acids (17, 18, 19).

Lin *et al.* (20, 21) found that the addition of substances such as cellulose, pectin, or protopectin to the diet of rats increased the bulk of feces excreted without increasing the excretion of endogenous sterol. This suggests that the effect produced by cerebrosides cannot be accounted for simply by their low coefficient of digestibility. Also, Jones *et al.* (6) were unable to obtain evidence of specific bonding between cholesterol and phrenosin. It is possible, however, that the intestinal sterols dissolve in the nonabsorbed lipid and thereby become unavailable for reabsorption.[7] It seems unlikely that esterification of the sterols affects their excretion significantly, since when cerebrosides were fed, the sterols were excreted almost entirely in the unesterified form.

Rosenheim and Webster (22) have provided evidence that dietary cholesterol is largely converted to coprosterol when it is fed with cerebrosides. If endogenously produced cholesterol were also converted to coprosterol in the presence of cerebrosides, this could offer an explanation of the increased sterol excretion, since coprosterol is poorly absorbed from the

[7] The author is indebted to Dr. N. S. Radin for this suggestion.

TABLE 3. EFFECT OF CEREBROSIDE PREPARATIONS ON FECAL STEROL EXCRETION *

Preparation	Percentage in Diet	Body Weight Gain of Rats	Fecal Sterol †	
		g./day	*mg./g. feces*	*mg./rat/day*
Recrystallized Cerebrosides				
Fraction 1	5	5.9	8.6	13
Fraction 2	5	6.0	13.8	23
Fraction 3	5	6.0	13.3	21
Fraction 4	5	6.0	14.8	21
Fraction 5	5	6.4	18.0	27
Residue	5	5.6	10.6	13
Recrystallized Methyl Esters of Cerebroside Fatty Acids				
Iodine value 3.6	5	4.9	5.1	8
Iodine value 54.3	5	4.4	6.9	10
Fractionally Distilled Methyl Esters of Cerebroside Fatty Acids				
Fraction 1	5	3.6	5.4	7
Fraction 2	5	3.8	6.4	8
Fraction 3	5	1.7	6.0	8
Fraction 4	5	4.9	4.7	7
Fraction 5	5	3.9	5.7	9
Fraction 6	5	2.9	7.3	11
Residue	5	3.7	6.1	10
Ceramide	5	1.0	8.0	9
Triacetylsphingosine	5	4.8	3.0	4

* The procedures used to obtain these preparations are described in the section on methods; analytical values are given in Tables 1 and 2.

† Digitonin-precipitable, Liebermann-Burchard-positive sterol calculated as cholesterol.

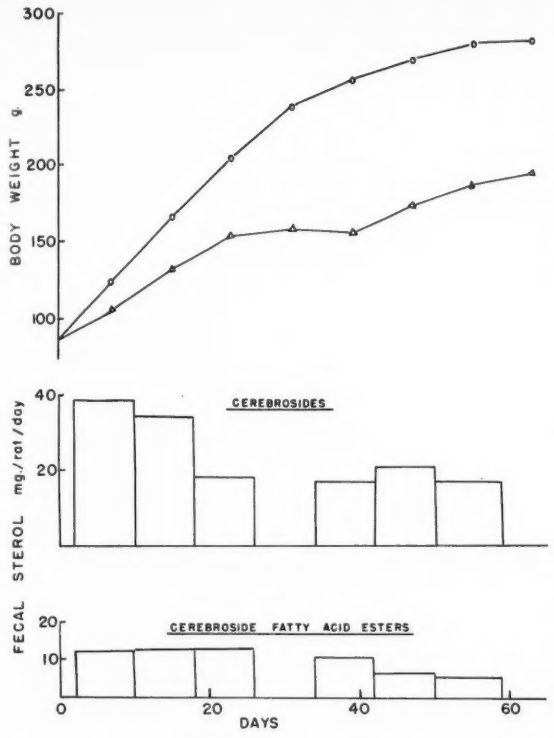

FIG. 2. The upper figure shows growth curves of rats fed synthetic diets containing 15% Wilson cerebrosides o—o—o or 15% mixed fatty acid esters obtained from the hydrolysis of Wilson cerebrosides Δ—Δ—Δ. The lower figures show the rate of excretion of digitonin-precipitable, Liebermann-Burchard-positive sterols by these rats during successive weekly intervals.

intestine. However, in our experiments, cholesterol appeared to be the main chromogenic sterol in the feces during the first week or two that the rats were fed cerebrosides, and although the pattern of fecal sterols changed later, this was associated with a reduction from the initial high levels of chromogenic sterol in the feces. These results indicate that the high level of chromogenic sterol in the feces during the first week of feeding cerebrosides is not a result of conversion of cholesterol to coprosterol in the intestine.

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REFERENCES

1. Carroll, K. K., and R. L. Noble. *Can. J. Biochem. and Physiol.* **34**: 981, 1956.
2. Jones, R. J., and O. K. Reiss. *Circulation* **12**: 496, 1955.
3. Jones, R. J., O. K. Reiss and E. L. Balter. *Circulation* **12**: 727, 1955.
4. Jones, R. J. *J. Lab. Clin. Med.* **47**: 261, 1956.
5. Jones, R. J., T. F. Keough, D. Cummings, and S. Kraft. *Circulation* **16**: 497, 1957.
6. Jones, R. J., O. K. Reiss, E. L. Balter, and L. Cohen. *Proc. Soc. Exptl. Biol. Med.* **96**:442, 1957.
7. Carter, H. E., W. J. Haines, W. E. Ledyard, and W. P. Norris. *J. Biol. Chem.* **169**: 77, 1947.
8. Fairbairn, N. J. *Chem. & Ind. London*, 86 (1953).
9. King, E. J. *Biochem. J.* **26**: 292, 1932.
10. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187**: 97, 1950.

TABLE 4. EFFECT OF 5 PER CENT CEREBROSIDES (WILSON) ON ABSORPTION OF EXOGENOUS CHOLESTEROL *

	Food intake per 24 hrs.	Cholesterol					
		Amount in diet	Intake per 24 hrs.	Fecal output per 24 hrs.	Output in excess of control	Amount absorbed	Percentage absorbed
	g.	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	
First collection period	14.9	0	0	9.4	—	—	—
	15.2	0.25	38.0	35.9	26.5	11.5	30
Second collection period	16.3	0	0	11.4	—	—	—
	17.8	0.25	44.5	38.3	26.9	17.6	40

* Results were obtained with groups of three male rats fed synthetic diets with and without added cholesterol. After a 2-day orientation period, the food intake was measured and the feces collected for two successive 7-day periods. The diets lacking cholesterol were used as controls to assess the excretion of endogenous cholesterol. The values for food intake and fecal excretion are divided by three, so that the results are expressed in terms of a single rat.

11. Carter, H. E., and Y. Fujino. *J. Biol. Chem.* **221:** 879, 1956.
12. Klenk, E. In *The Lipids,* H. J. Deuel, Jr., New York, Interscience Publishers, Inc., 1951, vol. 1, p. 492.
13. Carter, H. E., W. P. Norris, F. J. Glick, G. E. Phillips, and R. Harris. *J. Biol. Chem.* **170:** 269, 1947.
14. Carroll, K. K. *Can. J. Biochem. and Physiol.* **37:** 731, 1959.
15. Coleman, D. L., W. W. Wells and C. A. Baumann. *Arch. Biochem. Biophys.* **60:** 412, 1956.
16. Radin, N. S., and Y. Kishimoto. *Federation Proc.* **18:** 306, 1959.
17. Lin, T. M., E. Karvinen and A. C. Ivy. *Am. J. Physiol.* **183:** 86, 1955.
18. Lin, T. M., E. Karvinen and A. C. Ivy. *Proc. Soc. Exptl. Biol. Med.* **89:** 422, 1955.
19. Carroll, K. K. *Can. J. Biochem. and Physiol.* **37:** 803, 1959.
20. Lin, T. M., E. Karvinen and A. C. Ivy. *Am. J. Physiol.* **187:** 170, 1956.
21. Lin, T. M., E. Karvinen and A. C. Ivy. *Am. J. Physiol.* **188:** 66, 1957.
22. Rosenheim, O., and T. A. Webster. *Biochem. J.* **35:** 920, 1941.

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Studies on blood platelet phospholipids*

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SUMMARY

Column chromatography on silicic acid was carried out on phospholipids from acetone-dried, chloroform-extracted human blood platelets. Fractions recovered had the following components: Phosphatidylethanolamine (PE) plus phosphatidylserine (PS) plus inositol phosphatide, PE plus PS, lecithin plus sphingomyelin, and PS alone. Eluates containing only phosphatidylethanolamine and phosphatidylserine were rechromatographed to obtain more complete separation. The resulting fractions appeared to contain one component, but on hydrolysis of these materials it was apparent that each was still contaminated with small amounts of the other. However, on high dilution of each of these mixtures, blood thromboplastin formation was promoted; this suggested that both PE and PS could act as platelet thromboplastic factor *in vitro*. Inhibitory activity was not noted. The presence of choline phosphatides in the fractions resulted in loss of activity. Plasmalogens were identified and estimated to comprise 23 per cent of total platelet cephalins. They were more closely associated with phosphatidylethanolamine than phosphatidylserine. Lysolecithin could not be detected in the mixed platelet extract or the separated fractions.

In previous work (1) it was shown that phospholipids from acetone-dried, chloroform-extracted human blood platelets could be separated by silicic acid paper and column chromatography. Phosphatidylethanolamine (PE), phosphatidylserine (PS), lecithin, sphingomyelin, and inositol phosphatide were resolved by a combination of these procedures. Small amounts of phosphatidylserine obtained by column chromatography could replace the entire platelet phospholipid extract in the thromboplastin generation and the prothrombin consumption tests. Phosphatidylethanolamine preparations had the same thromboplastic activity but they contained phosphatidylserine. An unidentified fraction was obtained which also possessed coagulation properties; this was tentatively identified as a phosphatidic acid.

In the present study an attempt was made to obtain further separation of phosphatidylethanolamine from phosphatidylserine by altering the column size, mode and temperature of elution, and by rechromatography of combined fractions. Paper chromatographic identification of phosphatides including plasmalogen and lysolecithin was supplemented by hydrolysis of the lipids and examination for ethanolamine and serine bases (2).

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PE and PS, isolated by repeated column chromatography, appeared pure by paper chromatography of the intact phosphatides. However, when these materials were hydrolyzed and their bases identified, it appeared that each component was still contaminated by the other. Other workers[1] (3) also have noted that PE and PS could not be obtained in chemically pure form from silicic acid columns. However, Marinetti *et al.* (4) recently reported recovery of pure PS by silicic acid chromatography of a relatively uncontaminated preparation of Folch fraction 3 from pig brain.

METHODS

Preparation of Mixed Phospholipid Extracts. Blood collection and processing was carried out by methods previously described (1). After venesection, however, all operations were performed in a cold room (8°C). Five hundred ml. of blood from a donor with a normal platelet count yielded 8 to 15 mg. of mixed platelet phospholipids.

Column Chromatography. The apparatus used has been described (1) and the procedure was basically that of Hirsch and Ahrens (5). Columns 37 cm. long and 1.2 cm. internal diameter were packed dry with

[1] D. J. Hanahan. Personal communication, 1958.

18 g. of silicic acid.[2] Mixed platelet lipid (311 mg., 10.8 mg. phosphorus) was charged in 3.5 ml. of petroleum ether (b.p. 30°-60°C), and the first elution was made with 220 ml. of 1 per cent ethyl ether in petroleum ether, followed sequentially by 200 ml. 25 per cent ethyl ether in petroleum ether, and 350 ml. ethyl ether. These elutions were completed in 48 hours.

The phospholipids were then eluted with 450 ml. methanol in 2 ml. fractions. To preserve column integrity methanol was added very slowly to the ether at the head of the column. The phospholipid elution carried out at 20°C was completed in 24 hours but fractions were removed every half hour to storage at —20°C.

For study each fraction was taken to dryness under purified nitrogen and reconstituted in 4 ml. petroleum ether. Petroleum ether was selected because it was a more convenient solvent for paper chromatography and some of the phosphatides in the separated state were not completely soluble in methanol. Unless otherwise indicated, all calculations were based on the original 2 ml. fraction.

Rechromatography of Phosphatidylethanolamine and Phosphatidylserine Fractions. In an attempt to separate PE and PS, fraction numbers 25 to 30 (Fig. 1) were combined, suspended in 1 ml. of 20 per cent methanol in chloroform and rechromatographed at 10°C on 8 g. of silicic acid.[2] The latter was dried overnight at 115°C and washed in the column with 20 per cent methanol in chloroform for 15 hours prior to charging in the usual manner. Successive elutions were performed on the same charge as follows: 1000 ml. methanol : chloroform 1 : 4; 240 ml. methanol : chloroform 2 : 3; and 200 ml. methanol : chloroform 4 : 1 (v/v). One-ml. fractions were collected, evaporated, and resuspended in 2 ml. petroleum ether. Calculations were based on the original 1 ml. collected unless otherwise stated. The tube contents were tested for ninhydrin reactivity (4 to 5 μl. aliquots).

The concentrations of eluting solvents were varied since it was not known which proportion would most effectively partition these two fractions. However, it was found that the methanol : chloroform 1 : 4 eluted all the PE and PS applied to the column. After the first few tubes containing the column wash were discarded, the next 42 fractions, ninhydrin positive, were taken for further study. Two successive tubes were pooled for hydrolysis and paper chromatography, while the third was divided into two equal portions for the thromboplastin generation tests and phosphorus determinations.

Paper Chromatography. Usually 10 μl. from each fraction were applied to silica impregnated Whatman No. 1 filter papers, 6.5 cm. wide and 35 cm. long. Large concentrations of phospholipid were used in many instances in order to avoid missing small amounts of contaminant. When abrupt changes in the chromatographic patterns were found (appearance or disappearance of components), the run was repeated twice. Other details were the same as previously described (1) for the system using diisobutyl ketone : acetic acid : water (40 : 25 : 5, v/v/v) (6). Wet chromatograms were promptly viewed under ultraviolet light. Stains for ninhydrin and choline were used for confirmation and further identification of spots (1).

For mixed extracts and the diisobutyl ketone : acetic acid : water system (with 0.001 per cent Rhodamine 6G dye as the stain) (6), a minimum of 1.2 μg. of lipid phosphorus was required for adequate chromatograms. For detection of a single spot, at least 0.2 μg. of lipid phosphorus was required.

Hydrolysis. Estimation of ethanolamine and serine was carried out by alkaline hydrolysis and the formation of a colored derivative with dinitrofluorobenzene (2). The sensitivity of the method for measuring ethanolamine was increased by the use of microcuvettes in a Beckman spectrophotometer. Phosphorus was analyzed in duplicate by the method of Dryer *et al.* (7).

Aldehyde was determined in duplicate on 0.5 ml. aliquots with Schiff's reagent (6) and palmitaldehyde[3] as the standard. For the detection of acetal phosphatides on paper chromatograms, 2,4-dinitrophenylhydrazine in 3N HCl was used (6).

Thromboplastin generation tests (8) were performed on 0.5 ml. aliquots transferred to a test tube and taken to dryness under nitrogen. A suspension in 0.3 ml. of imidazole buffer (pH 7.35, 270 milliosmols per liter) was made immediately. This was arbitrarily designated the "undiluted" tube. The lipid was dispersed by rapid agitation with a pointed glass rod. The emulsions were grossly turbid in direct proportion to the lipid content, and settling was not observed after standing for more than 6 hours. All fractions had the same dispersion characteristics regardless of clotting activity. In the late methanol fractions in which only small amounts of lipid were present, several successive tubes were pooled for adequate study. To determine whether any lipid with clotting activity remained on the silicic acid columns after completion of the elu-

[2] Bio-Rad Laboratories, Richmond, Calif. Batch SAB-8.

[3] K & K Laboratories, Long Island City 1, N.Y. The material was supplied as the sodium bisulfite salt. The free aldehyde was liberated by the addition of excess 3N HCl to 5 g. of the salt followed by filtration and repeated washing with distilled water.

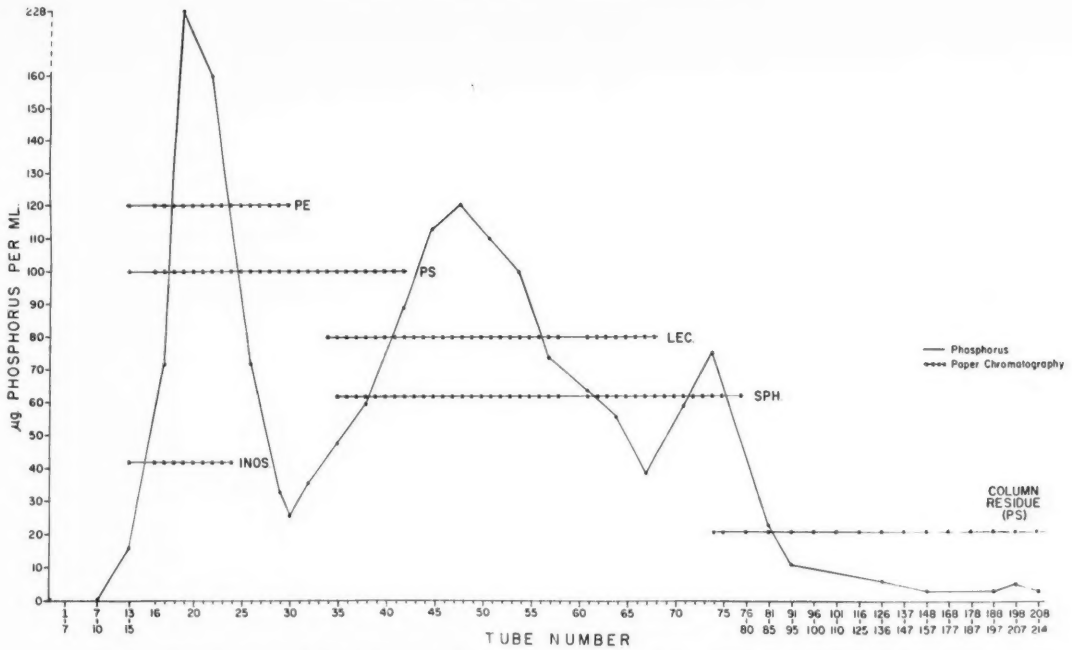

FIG. 1. Phosphorus analysis and paper chromatographic identification of original fractions eluted with methanol. Material under the first peak was ninhydrin and Schiff positive beginning with tubes 13 to 15, and had thromboplastic activity. The second peak was choline positive, ninhydrin negative, and contained Schiff reacting material in progressively decreasing quantities. These fractions did not promote thromboplastic activity. In the region of tube 75 the choline reaction became negative and clotting activity reappeared. In the paper chromatographic study, it is to be noted that the early appearance of lecithin and sphingomyelin interfered with recovery of pure phosphatidylserine. The "column residue" was almost pure phosphatidylserine. Fractions 25 to 30 were taken for rechromatography.

tions, the columns were unpacked, the silicic acid suspended in a large volume of methanol, and filtered. The filtrate was evaporated under nitrogen and tested in the thromboplastin generation test.

The prothrombin consumption tests were performed in duplicate, using plasma from the same donor throughout the study. A 12-hour fast preceded each blood collection. Platelet-free "native" plasma (no anticoagulant) was prepared as previously described (1), except that the entire procedure was carried out in a cold room. Buffer blanks and plasma alone in duplicate were used as controls. Serum prothrombin times were determined after 2 hours' incubation by the method of Ware and Stragnell (9).

The late fractions from the original methanol elution were investigated for hemolytic activity in the following manner: Approximately 50 µg. of phospholipid was evaporated to dryness and resuspended in 0.3 ml. of imidazole buffer. Dilutions were carried out in a rack of five Kahn tubes. Finally, 0.1 ml. of 1 per cent suspension of washed sheep red blood cells (10) was placed in each tube. Gross examination for hemolysis was carried out after 2 hours' incubation at 37°C.

Upon completion of coagulation tests, phosphorus analyses, hydrolysis studies, and hemolysis tests, the last 75 methanol fractions were pooled and evaporated to dryness. One mg. of material was obtained, which was analyzed for ninhydrin positive material and phosphorus.

RESULTS

Column Chromatography. The fractions eluted with ethyl ether were ninhydrin negative and gave no Liebermann-Burchard reaction.[4] A faint trace of phosphorus was found in an occasional tube.

Results of phosphorus analysis on fractions initially eluted with methanol are given in Figure 1. As shown on the abcissa, the contents of certain tubes were pooled to facilitate their study. The first peak represents the cephalin fraction and the second peak lecithin and sphingomyelin. The "column residue" refers to material eluted after the second peak began to approach baseline levels. Recovery of phosphorus from

[4] The early supernatants obtained when the whole platelets were washed in acetone gave a positive Lieberman-Burchard reaction.

the column in the original methanol elution was 92.5 per cent (10 out of 10.8 mg.).

The milligram of lipid in the combined last 75 methanol fractions was found to contain 3.8 per cent phosphorus. Forty per cent was ninhydrin reacting material, a finding that invalidates the previous tentative identification as phosphatidic acid (1).

Paper Chromatography. Results of paper chromatograms on the individual original methanol fractions are given in Figure 1. The "column residue" had the following characteristics: It migrated as a well-defined single spot, and the staining reaction with Rhodamine G was bright blue. The R_f value was 0.6 to 0.8, the ninhydrin reaction was positive, choline was not detected, and there was a faint positive reaction with 2,4-dinitrophenylhydrazine. These properties resembled phosphatidylserine, but not phosphatidic acid or lysolecithin. The material also possessed thromboplastic activity.

The pooled original methanol fractions 195 to 207 and 210 to 214 did not exhibit hemolytic activity. Thus lysolecithin could not be found by the methods employed in this study, although this lipid has been described in serum and red cells (11).

TABLE 1. PHOSPHORUS ANALYSES AND PAPER CHROMATOGRAPHY OF RECHROMATOGRAPHED FRACTIONS

Tube Number	*μg. Phosphorus per ml.*	*Paper Chromatography*	
		PE	*PS*
18	—	+	
24	9.3	+	
27	5.4	+	
30	3.3	+	+
33	2.6	+	+
36	2.0	+	+
39	1.6	+	+
40-41	—		+
42-51	7.5		+

In Table 1 the results of rechromatography of the combined PE and PS fractions are shown. The solvent pair, methanol : chloroform 1 : 4 eluted all the ninhydrin positive material. Initially, phosphatidylethanolamine was eluted as a pure fraction. This was followed by a mixture, and finally by pure phosphatidylserine.

Thromboplastin Generation Test. (See Fig. 2.) A fraction was considered active if the thromboplastin time was within 10 seconds of control. All the fractions could be diluted about 75 times and still remain within 10 seconds of control. The results through tube 24 represent the activity of the cephalin group. Tubes 25 to 30 show the activity of phosphatidylethanolamine and phosphatidylserine. The thromboplastin time in tubes 31 to 33 was accounted for by phosphatidylserine. The appearance of lecithin and sphingomyelin in fraction 34 coincided with an abrupt loss of thromboplastic activity which persisted through fraction 76, when sphingomyelin became undetectable. At this point clotting activity returned. The thromboplastin times of the column residue were not as consistently close to control as the early fractions, but most values approximated that range of activity.

The effect of purification was reflected in the phosphorus values of the active fractions; that is, when the fractions were used, much less phospholipid was required to give the same thromboplastic activity as when mixed platelet lipid was employed. When whole platelet phospholipid extract was used in the thromboplastin generation test, it was found that optimal activity was obtained when the concentration of phosphorus in the reagent tube was 30 μg. per ml. In the original methanol fractions (tubes 31 to 33, for example) phosphorus values of 0.5 μg. per ml. were calculated at optimal clotting ranges. The same situation was noted in the rechromatographed fractions which were identified as PE. Phosphorus concentration averaged 0.2 μg. per ml. as compared to 30 μg. per ml. of mixed material.

When the rechromatographed chloroform : methanol fractions were tested in the thromboplastin generation test, activity within control range was obtained uniformly with undiluted and 1 : 1 diluted materials. The tubes containing primarily PE and primarily PS had the same order of activity as those which contained mixtures of the two lipids.

Thromboplastin generation tests were repeated on six fractions after 5 months' storage in petroleum ether at —20°C. There was no change in the clotting activity as measured by this test. Fractions stored in buffer at —20°C were also stable after 5 months.

The ethyl ether eluates of the original column separation were tested for thromboplastic activity and were found to be inert. The material removed from the silicic acid packing after chromatography also had no clotting activity.

Prothrombin Consumption Tests. (See Fig. 2.) The results with the original methanol column fractions indicated that as the cephalin fraction was purified the prothrombin consumption increased. The early pooled "column residue" tubes were able to increase prothrombin consumption, but the later fractions did not produce as marked an increase despite the fact

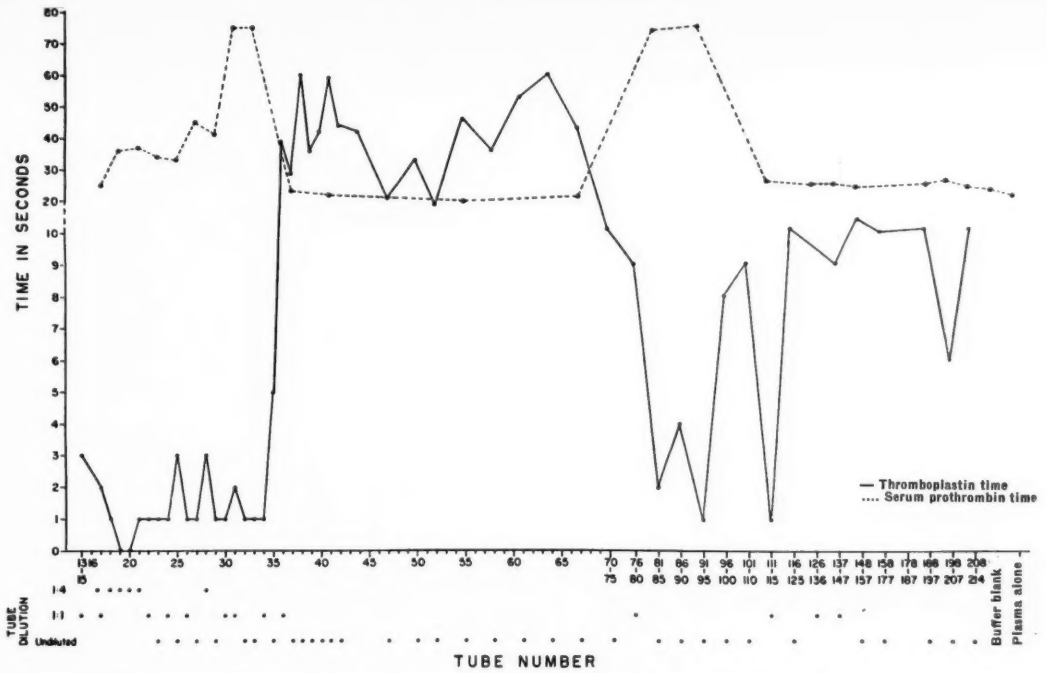

FIG. 2. Results of thromboplastin generation and prothrombin consumption tests on original methanol fractions. The points connecting the solid lines represent the results when the indicated fraction was used as platelet reagent in the thromboplastin generation test. The difference between the clotting time of the fraction and the brain cephalin control at the end of 6 minutes' incubation is designated as the thromboplastin time. Low numbers on the ordinate represent activity. The figures beneath the abcissa scale represent the dilution at which the tube showed the maximal activity indicated in the chart. The points between the broken lines represent the serum prothrombin time (prothrombin consumption) of the original methanol fractions. A high value on the ordinate scale indicates ability to increase the prothrombin consumption of platelet-free native plasma.

that they were active in the thromboplastin generation test. (It is possible that the 1 : 4 dilution of the small amount of lipid in these tubes was insufficient to increase prothrombin consumption.) In these studies, as in the thromboplastin generation test, the amount of purified material which gave the same prothrombin consumption as mixed platelet lipid was quite small. After dilution, the 0.1 ml. of mixed lipid added to the plasma contained a total of 382 μg. of phospholipid, whereas the phosphatidylserine fractions contained an average of 9 μg. and active column residue fractions contained 0.13 μg. of phospholipid.

Plasmalogen. Quantitative aldehyde studies are shown in Figure 3. The bulk of the plasmalogen was in the cephalin fraction. Approximately 23 per cent of the phosphorus in tubes 16 to 31 was present as plasmalogen[5] (12). As reported by others (13), the ethanolamine plasmalogens predominated; indeed, the drop in plasmalogen value coincided with the disappearance of phosphatidylethanolamine. In the rechromatographed methanol : chloroform fractions plasmalogen was noted only in the tubes where PE predominated. Tube 21 contained a total of 1.2 μg. In the other rechromatographed fractions sufficient material for quantitative study was not available, but they were qualitatively Schiff positive through tube 33.

Hydrolysis. Results obtained with the original methanol fractions are given in Figure 4. In the early tubes large amounts of PE and PS were found, with PS predominating after fraction 31. In every subsequent tube PS was found along with smaller amounts of PE, even in the column residue.

The same general pattern was obtained with the rechromatographed eluates (Fig. 5), that is, PE was the major component at first, followed by a mixture of PE and PS and then persistence of PS. Pooled fractions 42 to 51, which were not hydrolyzed, were identified on paper as PS. It should be mentioned that the over-all error of the method of hydrolysis would make the figures obtained for serine in the rechromatographed

[5] Since the molar ratio of phosphorus to aldehyde is 1, the following formula was used:

$$\frac{\text{moles of aldehyde}}{\text{moles of phosphorus}} \times 100 = \begin{matrix}\text{phosphorus present as}\\ \text{plasmalogen as a percentage}\\ \text{of the total phosphorus.}\end{matrix}$$

TABLE 2. CORRELATION OF PHOSPHORUS CONTENT WITH DNP ETHANOLAMINE AND DNP SERINE IN RECHROMATOGRAPHED FRACTIONS *

Fraction	A DNP Serine (analyzed)	B P Content of Phosphatidylserine (calculated)	C DNP Ethanolamine (analyzed)	D P Content of Phosphatidylethanolamine (calculated)	E Sum of Columns B & D	F Total P (analyzed)
	μg./ml.	μg./ml.	μg./ml.	μg./ml.	μg./ml.	μg./ml.
22 & 23	5.1	1.5	12.6	6.4	7.9	
24						9.2
25 & 26	5.1	1.5	10.6	5.4	6.9	
27						5.4
28 & 29	5.0	1.5	5.2	2.6	4.1	
30						3.2
31 & 32	4.5	1.3	3.1	1.6	2.9	
33						2.6
34 & 35	3.3	1.0	1.9	1.0	2.0	
36						2.0
37 & 38	1.8	0.5	1.5	0.7	1.2	
39						1.6
40 & 41	2.1	0.6	0.7	0.4	1.0	

* Figures in column B are obtained by multiplying the result in column A by 31/105. Similarly, the values in column D are derived by multiplying the result in column C by 31/61. The totals in column E represent the values that should be found if all the phosphorus is derived from PE and PS. Column F is the actual amount of phosphorus in the adjacent tube.

fractions 10 to 15 and 16 to 17 doubtful, since the readings were quite close to the blank. However, the low ethanolamine figures are significant because of more sensitive methods of measurement.

Correlation of Phosphorus Content with Dinitrophenylhydrazine Ethanolamine and Dinitrophenylhydrazine Serine Analysis.[6] In Table 2 evidence is presented that the phosphorus measured was derived from phospholipid. A shortcoming of these calculations is that the phosphorus figure was taken from the adjacent tube because of insufficient material for determinations on every fraction. Column E represents the sum of the calculated amount of phosphorus if it were derived only from PE and PS. In column F the total amount of phosphorus in the adjacent fraction is given. The close correspondence of values in columns E and F suggests that all phosphorus could be accounted for as PE and PS phosphorus.

[6] Calculating on the basis of a nitrogen to phosphorus ratio of 1:1, one mole of PS contains one atomic weight of phosphorus and will yield after hydrolysis one mole of serine. Similarly, one mole of PE contains one atomic weight of phosphorus and will yield after hydrolysis one mole of ethanolamine. Therefore:

$$\mu\text{g./ml. P} = \frac{\text{at. wt. P}}{\text{m. wt. serine}} \times \mu\text{g./ml. serine}$$

and

$$\mu\text{g./ml. P} = \frac{\text{at. wt. P}}{\text{m. wt. ethanolamine}} \times \mu\text{g./ml. ethanolamine}$$

DISCUSSION

When mixed platelet phospholipid extracts were eluted from silicic acid columns with methanol, the first fractions possessed thromboplastic activity. This early component was a mixture containing phosphatidylethanolamine, phosphatidylserine, and inositol

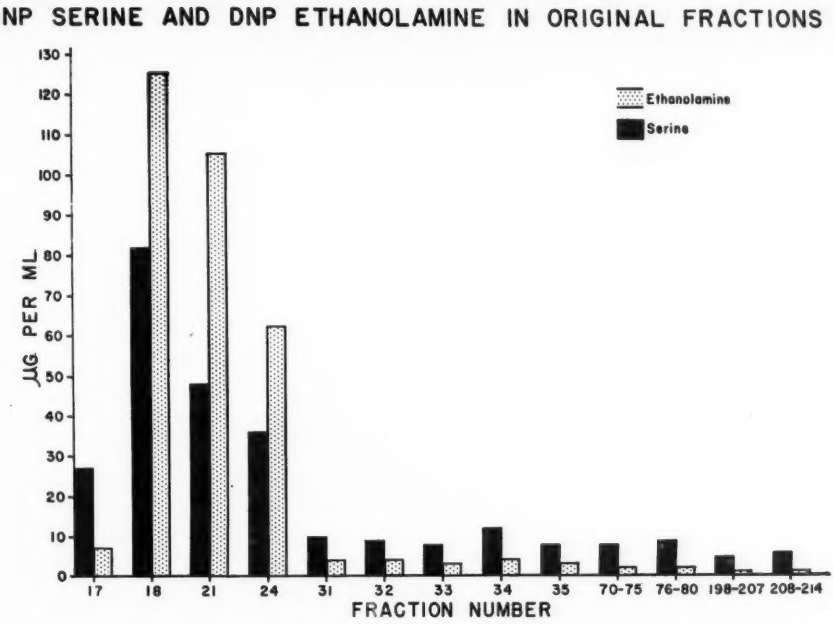

FIG. 4. Hydrolysis studies on original silicic acid column methanol eluates. Fractions 31 to 33, 198 to 207, and 208 to 214 appeared on paper chromatography to be phosphatidylserine; however, as seen above, PE was present in these fractions.

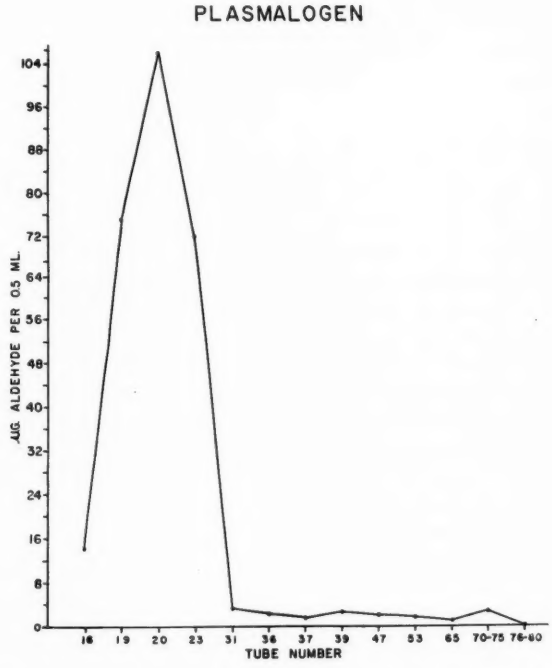

FIG. 3. Aldehyde determinations with Schiff's reagent on original silicic acid column methanol eluates. High values follow the distribution of PE rather than PS.

phosphatide. As the elution progressed, inositol phosphatide became undetectable and its disappearance was followed by that of phosphatidylethanolamine, leaving PS almost free of other phosphatides. The rapidity with which the choline phosphatides appeared (Fig. 1) may have been a function of temperature, since a higher yield of PS was obtained (1) by chromatography at 8°C than at 20°C. The appearance of lecithin and sphingomyelin coincided with a loss of coagulation activity. Elution beyond the point of complete recovery of lecithin and sphingomyelin again gave PS, which promoted thromboplastin formation. Thus there were two sources of phosphatidylserine in this initial separation. A second chromatographic procedure of the eluates containing PE and PS with methanol : chloroform 1 : 4 produced chromatographically pure phosphatidylethanolamine (Table 1).

Fractions which appeared to be homogeneous, judged by paper chromatography of the intact phosphatides, were found to be mixtures when hydrolyzed. Thus greater sensitivity is afforded by evaluation of hydrolysis products.

Positive statements cannot be made concerning the individual role of platelet PE and PS in blood thromboplastin formation. However, the following observations are mentioned: In the original methanol fractions (31 to 33 and column residue, Fig. 1) in which

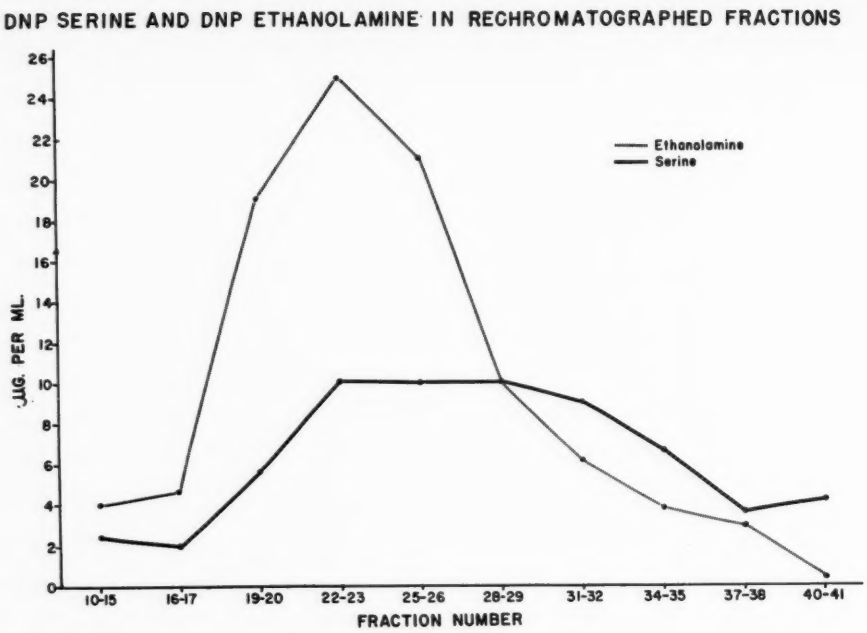

FIG. 5. Hydrolysis results on the rechromatographed fractions (methanol: chloroform 1:4). Although there was a good yield of PE, it could not be freed of PS. Again, paper chromatography revealed only the predominating phosphatide (see Table 1).

PS existed in highly purified form, clotting activity was noted at dilutions at which PE contamination was undetectable. The same phenomenon was noted in rechromatographed fraction number 18 (Fig. 5), which contained PE contaminated with trace amounts of PS. It is possible that PE and PS are interchangeable as platelet thromboplastic factors *in vitro*. However, evidence is insufficient to be conclusive.

Thromboplastic activity has been noted with PE in other laboratories (14 to 17) but PS was found to be inhibitory (17[7]). On the other hand, activity with PS has been noted at small concentrations (18) or under special circumstances (17, 19, 20). In our hands inhibitory activity occurred only at high concentrations (1), and the presence of lecithin (obtained from columns or purified commercial lecithin) caused a loss of thromboplastic activity. Possible explanations for some of these discrepancies are: (*a*) materials considered to be homogeneous actually were contaminated, (*b*) in the course of isolation the fatty acid component of the phospholipid molecule was oxidized, resulting in a reduction or loss of thromboplastic activity, (*c*) different systems were employed to study thromboplastic activity, and (*d*) under certain circumstances PE and PS might both be capable of promoting thromboplastin formation.

Plasmalogens are phospholipids which will release higher fatty aldehydes when treated with mercuric salts or weak mineral acids (21). They are known constituents of nervous and muscular tissue and sperm (22). In this study plasmalogens were demonstrated in blood platelets and composed 23 per cent of the cephalin fraction. Most of the platelet plasmalogen was apparently of the ethanolamine type. Small amounts were also detected in the choline fractions. The presence of these phosphatides in fractions with thromboplastic activity is of interest, since the role of plasmalogens in lipid metabolism is not known (23). It is reported that brain plasmalogens do not have anticoagulant activity (24).

[7] Barkhan and Silver recently reported (American Society of Hematology Meeting, November 24, 1959, St. Louis, Mo.) acceleratory activity with low concentrations of PS fractions from brain.

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REFERENCES

1. Marcus, A. J., and T. H. Spaet. *J. Clin. Invest.* **37:** 1836, 1958.
2. Axelrod, J., J. Reichenthal and B. B. Brodie. *J. Biol. Chem.* **204:** 903, 1953.
3. Olley, J. *Federation Proc.* **16:** 845, 1957.
4. Marinetti, G. V., J. Erbland and E. Stotz. *Biochim. et Biophys. Acta* **30:** 41, 1958.
5. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
6. Marinetti, G. V., J. Erbland and J. Kochen. *Federation Proc.* **16:** 837, 1957.
7. Dryer, R. L., A. R. Tammes and J. I. Routh. *J. Biol. Chem.* **225:** 177, 1957.
8. Spaet, T. H. *Am. Practitioner and Dig. Treatment* **7:** 403, 1956.
9. Ware, A. G., and R. Stragnell. *Am. J. Clin. Pathol.* **22:** 791, 1952.
10. Phillips, G. B. *Proc. Natl. Acad. Sci. U.S.* **43:** 566, 1957.
11. Phillips, G. B., and N. S. Roome. *Proc. Soc. Exptl. Biol. Med.* **100:** 489, 1959.
12. Gray, G. M., and M. G. Macfarlane. *Biochem. J.* **70:** 409, 1958.
13. Lovern, J. A. *The Chemistry of Lipids of Biochemical Significance*. New York, John Wiley & Sons, Inc., 1955, p. 21.
14. O'Brien, J. R. *J. Clin. Pathol.* **9:** 47, 1956.
15. Rouser, G., S. G. White and D. Schloredt. *Biochim. et Biophys. Acta* **28:** 71, 1958.
16. Rouser, G., and D. Schloredt. *Biochim. et Biophys. Acta* **28:** 81, 1958.
17. Barkhan, P., M. J. Silver and L. M. Tocantins. *Federation Proc.* **18:** 8, 1959.
18. Troup, S. B., and C. F. Reed. *J. Clin. Invest.* **37:** 937, 1958.
19. Therriault, D., T. Nichols and H. Jensen. *J. Biol. Chem.* **233:** 1061, 1958.
20. Rapport, M. M. *Nature* **178:** 591, 1956.
21. Rapport, M. M., and R. E. Franzl. *J. Biol. Chem.* **225:** 851, 1957.
22. Hanahan, D. J. *Federation Proc.* **16:** 826, 1957.
23. Thannhauser, S. J. *Lipidoses*. New York, Grune & Stratton, Inc., 1958, p. 18.
24. Turner, D. L., M. J. Silver and L. M. Tocantins. *Arch. Biochem. and Biophys.* **77:** 249, 1958.

Notes on Methodology

Gas-liquid chromatography of fatty acid methyl esters: the "carbon-number" as a parameter for comparison of columns

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► Because of the slight but perceptible volatility of stationary phases, absolute retention times of components eluted from gas-liquid chromatographic columns decrease steadily as the column is repeatedly used. This is particularly the case for the polar polymers now customarily used for the separation of unsaturated fatty acid esters in lipid analysis. Many other factors (e.g., small differences in pressure, temperature, or other operating conditions of the column) also affect the absolute retention time of a given methyl ester. For these reasons it has become the practice to quote relative retention times referred to one component of the mixture, e.g., palmitate or stearate. We have found it more convenient to express the elution sequence in different terms, obtained by the following procedure.

The retention times of the saturated straight-chain esters, at least four of which are usually present in natural mixtures, are plotted against chain-length on semilogarithmic paper and the best straight line joining them is constructed. (Identification of these peaks, usually self-evident, can be confirmed if necessary by the addition of known esters to the mixture under investigation.) A value corresponding to the retention time of any other peak can then be read off from the graph, and gives the chain length of the hypothetical saturated straight-chain ester which would be eluted at that point. The figure so obtained we designate the "carbon-number" of the ester on the stationary phase in question. Saturated esters with a straight chain have integral carbon-numbers, e.g., 15.0, 17.0 (so that in such cases the phrase has its conventional meaning), whereas esters with a branched chain and unsaturated esters have, in general, non-integral C-numbers. The carbon-number is characteristic of a particular ester on a particular stationary phase. It is, like relative retention volume, dependent on the temperature at which the column is run, but is sufficiently constant over small temperature ranges (at least 20°C) to allow comparison with other runs on the same stationary phase (including those of other authors) made in the same temperature region.

It is the practice in this laboratory to determine the fatty acid composition of fractions (phospholipids, free fatty acids, sterol esters, and glycerides) obtained by the analysis of lipids from human arteries (1) by running each sample on two columns, one containing Apiezon "L" and the other a copolymer of maleic acid, adipic acid, and ethylene glycol (MAE). The carbon-numbers of commonly occurring esters on these two stationary phases are shown in Table 1. For comparison, carbon-numbers calculated from the data of other authors are also shown. Table 1 illustrates the well-known fact that unsaturated esters are eluted before the corresponding saturated ester on Apiezon, after it on a polar stationary phase.

TABLE 1. Comparison of Carbon-Numbers (Hypothetical Chain Lengths) of Methyl Esters of Commonly Occurring Fatty Acids on Polar and Nonpolar Stationary Phases

Methyl Ester	MAE * (185°C)	Apiezon "L" † (203°C)	Apiezon "M" § (197°C)	Apiezon "M" ‖ (197°C)	Apiezon "L" # (240°C)
Palmitoleate	16.4	15.75	15.7	15.75	15.75
Oleate	18.3	17.65	17.6	17.7	17.7
Linoleate	18.9	17.5	17.5	17.4	17.6
Linolenate	19.65	17.55	—	17.42	17.6
Arachidonate	21.6	18.95	—	— ‡	19.0
Erucate	22.2	21.6	—	—	—

* Copolymer of maleic acid, adipic acid, and ethylene glycol 1 : 3 : 4, 1 g. per 4 g. Chromosorb®.

† 1 g. per 9 g. acid- and alkali-washed Celite 545®.

‡ The $\Delta^{8,11,14,17}$-tetraenoic C_{20} ester (isomer of arachidonate) investigated by these authors had a C-number of 18.85.

§ See Ref. 2. ‖ See Ref. 3. # See Ref. 4.

The behavior of saturated branched-chain esters is shown in Table 2. The figures in the last column are calculated from the relative retention times given by James and Martin (2). It can be seen that branched-chain esters are eluted before the corresponding straight-chain ester on both polar and nonpolar phases. Consideration of the carbon-numbers shows that the anteiso compounds are eluted slightly later, i.e., nearer the straight-chain ester than iso compounds.

These data enable us to assign structures to minor constituents of fatty acid mixtures with some measure of confidence: for example, the group of C_{15} acids which occurs with some frequency in natural mixtures.

TABLE 2. CARBON-NUMBERS OF BRANCHED METHYL ESTERS ON POLAR AND NONPOLAR STATIONARY PHASES

Methyl Ester	Carbon-Number on		
	MAE (185°C)	Apiezon "L" (203°C)	Apiezon "M"* (197°C)
4-Methylhexanoate (C_7 anteiso)	—	—	6.7
6-Methylheptanoate (C_8 iso)	—	—	7.45
6-Methyloctanoate (C_9 anteiso)	—	—	8.65
8-Methylnonanoate (C_{10} iso)	—	—	9.55
8-Methyldecanoate (C_{11} anteiso)	—	—	10.7
10-Methylundecanoate (C_{12} iso)	—	—	11.65
10-Methyldodecanoate (C_{13} anteiso)	—	—	12.7
12-Methyltetradecanoate (C_{15} anteiso)	—	—	14.7
14-Methylpentadecanoate (C_{16} iso)	15.6	15.7	15.65
14-Methylhexadecanoate (C_{17} anteiso)	16.7	16.7	16.7
16-Methylheptadecanoate (C_{18} iso)	17.6	17.6	—
16-Methyloctadecanoate (C_{19} anteiso)	18.8	18.7	—
20-Methylheneicosanoate (C_{22} iso)	21.6	21.55	—

* See Ref. 2.

Pentadecanoate has, of course, a C-number of 15.0 on both columns; in addition, there may occur a second peak on Apiezon, C-number 14.7, whose percentage corresponds to the sum of the percentages of the two peaks on MAE with C-numbers 14.5 and 15.4. These have been therefore tentatively identified as due to C_{15} branched and C_{15} (mono-) unsaturated esters, respectively. Support for this identification comes from the observation that in some natural mixtures only one of the C_{15} peaks occurs, having a C-number of 15.4 on MAE and 14.7 on Apiezon.

In this way several minor constituents of fatty acid mixtures have been provisionally identified on the basis of their two C-numbers alone, as shown in Table 3. The identification is based on the correlation of the results on MAE and Apiezon chromatograms of about 200 different lipid samples. The repeated appearance of peaks of the same relative magnitude on the two phases and with constant, appropriately related carbon-numbers establishes the existence and frequent occurrence of a definite unknown compound. It is then provisionally identified in accordance with the observed rules of behavior of branched and unsaturated esters; comparison with known samples where possible; and comparison with retention times reported in the literature, recalculated as carbon-numbers. Final confirmation of identity depends, of course, on further investigation of the isolated fraction or the use of hydrogenation, bromination, and ozonolysis procedures. For all the unsaturated acids shown in Table 3 the number of carbon atoms has been confirmed by fraction collection, hydrogenation, and rechromatography.

TABLE 3. PROVISIONAL IDENTIFICATION OF SOME PREVIOUSLY UNREPORTED FATTY ACIDS OCCURRING IN HUMAN ARTERY LIPIDS, BY DETERMINATION OF THE CARBON-NUMBERS OF THEIR METHYL ESTERS ON DIFFERENT STATIONARY PHASES

Observed Carbon-Number on		Tentative Identification
MAE *	Apiezon *	
14.5	14.7	C_{15} branched
15.0	15.0	C_{15} saturated
15.4	14.7	C_{15} monoenoic
16.6	16.7	C_{17} branched (? anteiso)
17.0	17.0	C_{17} saturated
17.3	16.7	C_{17} monoenoic
20.3	19.6	C_{20} monoenoic
20.7	19.4	C_{20} dienoic
21.1	19.2	C_{20} trienoic
22.0	22.0	C_{22} saturated
22.2	18.8	C_{20} pentaenoic
22.3	21.7	C_{22} monoenoic
23.0	23.0	C_{23} saturated
24.0	24.0	C_{24} saturated
24.3	23.6	C_{24} monoenoic

* Same conditions as in Table 1.

Carbon-numbers represent, of course, no difference in principle from relative retention times. We suggest, however, that they indicate more immediately and vividly the position in the elution pattern of a component under discussion (and therefore bring mistakes and anomalies more readily to light). Instead of being unattached numbers, they are closely connected with the chemical constitution of the esters; and finally, they make unnecessary the arbitrary choice of a reference compound to which retention times can be related. If retention times of fatty acid methyl esters were always expressed in this way, direct com-

parison of retention values on different phases and from different laboratories would be possible without recalculation and often from memory. Such comparisons have proved invaluable in this laboratory, and the results to which they have led will be reported subsequently.

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REFERENCES

1. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-van Houte, and C. M. van Gent. *Rec. trav. chim.* **78**: 794, 1959.
2. James, A. T., and A. J. P. Martin. *Biochem. J.* **63**: 144, 1956.
3. Stoffel, W., W. Insull, Jr. and E. H. Ahrens, Jr. *Proc. Soc. Exptl. Biol. Med.* **99**: 238, 1958.
4. Lipsky, S. R., J. E. Lovelock and R. A. Landowne. *J. Am. Chem. Soc.* **81**: 1010, 1959.

Determination of cholesterol and triglycerides in rat plasma*

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► By shaking human, dog, or rabbit plasma with chloroform in the presence of a synthetic zeolite, one obtains a phospholipid-free extract of cholesterol and triglyceride (1, 2). Cholesterol may then be quantitatively determined by any one of the standard procedures, while triglycerides are conveniently estimated by a determination of the glycerol after saponification (3). Similar experiments on rat plasma showed that the extraction of cholesterol or triglyceride was not quantitative unless the particle size of the zeolite was reduced to 80 to 100 mesh.

Methods. Extraction of lipids was carried out as described by Van Handel and Zilversmit (3), except that the extraction was continued overnight. Stock Zeolite[1] was divided into 40 to 79 mesh and 80 to 99 mesh sizes. Silicic acid column chromatography was applied to a purified chloroform extract as described by Van Handel and Zilversmit (3).

Cholesterol was analyzed after KOH saponification and extraction with petroleum ether. In addition, 1 ml. of plasma was analyzed by direct saponification, as in the method of Abell *et al.* (4).

The cholesterol content of petroleum ether extracts was determined by the method of Zak *et al.* (5). Triglycerides of chloroform extracts were measured by the method of Van Handel and Zilversmit (3). Complete removal of phospholipids was evidenced in all extracts by phosphorus analysis according to Bartlett (6). The results are shown in Table 1. A better extraction of cholesterol and of triglyceride was noted when the particle size of zeolite was decreased to 80 to 100 mesh. Differences like the one described here were not found in human, rabbit, or dog plasma. It would seem that at least two differences could explain these observations: (*a*) the cholesterol and triglycerides in rat plasma are chemically different from that found in other animals or (*b*) the cholesterol and triglycerides in rat plasma are more strongly bound to protein. The

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[1] Stock Zeolite-Taylor, obtained from W. A. Taylor Company, Baltimore, Md.

TABLE 1

Experiment	1		2		3		4	
Method	Cholesterol	Triglyceride	Cholesterol	Triglyceride	Cholesterol	Triglyceride	Cholesterol	Triglyceride
	mg./cc.	*mg./cc.*	*mg./cc.*	*mg./cc.*	*mg./cc.*	*mg./cc.*	*mg./cc.*	*mg./cc.*
Zeolite								
Stock	0.331	0.359	0.416	0.609	0.517	0.928	0.449	0.780
40–79 Mesh	0.451	0.485	0.465	0.718	0.562	0.966	0.512	0.780
80–100 Mesh	0.605	0.584	0.589	0.843	0.697	1.011	0.618	0.861
Silicic Acid Column	0.559	0.589	0.617	0.811	0.710	1.049	0.674	0.884
Abell	0.580	—	0.619	—	0.709	—	0.690	—

latter explanation seems the more reasonable, since coarse zeolite, when added to a chloroform extract of rat plasma, did not appear to adsorb cholesterol or triglyceride.

REFERENCES

1. Forbes, J. C. *J. Lab. Clin. Med.* **16**: 520, 1931.
2. Forbes, J. C., and H. Irving. *J. Lab. Clin. Med.* **16**: 909, 1931.
3. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50**: 152, 1957.
4. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195**: 357, 1952.
5. Zak, B., N. Moss, A. J. Boyle, and A. Zlatkis. *Anal. Chem.* **26**: 776, 1954.
6. Bartlett, G. R. *J. Biol. Chem.* **234**: 466, 1959.

Stabilization of polyester stationary phases for gas-liquid chromatography

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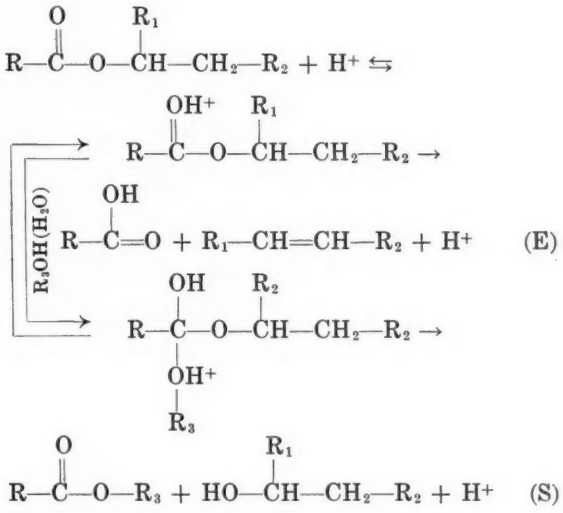
► The selection of stationary phases for gas-liquid chromatography at high temperature operation poses many problems. These problems center on the necessity for low vapor pressure, thermal stability, and a high separation factor of the stationary phases. With katharometer detectors the instability of the stationary phase frequently shows itself by the appearance of droplets ("bleed") from the column outlet or, more commonly, by the falling off of column efficiency. With the introduction of very sensitive ionization detectors (1, 2) the bleed is detected long before any physical signs from the column are noticeable. The manifestations are uncontrollable base line drifts and the development of "noise" at a level high enough to mask any peak and render further column operation impractical. Capillary columns (3) with ionization detectors are even more susceptible to stationary phase instability. This is because of their much greater sensitivity and the fact that there is only about 0.1 mg. of stationary phase per foot of column length (0.01 in. I.D. columns).



The development of polyester packings (4, 5, 6) has permitted the excellent fractionation of fatty acid methyl esters. However, different batches behave quite variably as regards stability (7). These esters could be expected to have free terminal hydroxyl groups or carboxyl groups. Generally, the residual condensing catalyst, i.e., *p*-toluenesulfonic acid, is also present. With hydrogen ions arising from either the catalyst present or the free carboxyl groups, it seems reasonable that acid-catalyzed ester interchange of the stationary phase within itself, solvolysis (S) with small amounts of water or alcohols in samples or elimination reactions (E) could occur. These reactions would split the polymer and eventually give rise to low-boiling products.

To test the idea that hydrogen ions may be responsible for degeneration of the polymer, alcoholic solutions were treated on an ion exchange resin, Duolite A-4,[1] a weak base. Three hundred g. of polymer (6) prepared from succinic acid, diethylene glycol, and diglycerol (*p*-toluenesulfonic acid as catalyst) was dissolved in 3 l. of 70 per cent ethanol and passed through a Duolite A-4 ion exchange resin in a column 7.5 cm. × 70 cm. About 10 l. of 70 per cent alcohol was used to wash the resin from the column. The alcohol was removed *in vacuo* and the residual oil heated at 50° C and 5 μ. pressure overnight. Columns

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

[1] Mention of specific products does not imply endorsement by the U.S. Department of Agriculture over others of similar nature not mentioned.

packed with this material showed very little bleeding and column life was extended markedly.

Columns prepared from stationary phases which were similarly treated, except for passage through the ion exchange resin, showed the instability otherwise regularly observed. However, when a column begins to show deterioration (excessive noise level of the background) it is damaged beyond recovery and should be repacked.

Care should also be taken to remove the acid from packing supports where they are used. It is customary to treat the Celite or C_{22} brick dust with hydrochloric acid, wash with water, and dry. Some improvement in the supports can be obtained by an aqueous ammonia treatment rather than aqueous sodium hydroxide (8) following the acid and water wash. Afterward the support is carefully washed and dried.

REFERENCES

1. Lovelock, J. E. *Nature* **182**: 1663, 1958.
2. Harley, J., W. Nel and V. Pretorius. *Nature* **181**: 177, 1958.
3. Golay, M. J. E. In *Gas Chromatography*, edited by V. J. Coates, H. J. Noebels, and I. J. Fagerson, New York, Academic Press, Inc., 1958.
4. Lipsky, S. R., and R. A. Landowne. *Biochim. et Biophys. Acta* **27**: 666, 1958.
5. Orr, C. H., and J. E. Callen. *J. Am. Chem. Soc.* **80**: 249, 1958.
6. Craig, B. M., and N. L. Murty. *Can. J. Chem.* **36**: 1297, 1958.
7. Horning, E. C., E. A. Moscatelli and C. C. Sweeley. *Chem. & Ind. London*, 751 (1959).
8. James, A. T., A. J. P. Martin and G. H. Smith. *Biochem. J.* **52**: 238, 1952.

New Methods

► **New Methods is a listing of recent and pertinent references to analytical lipid methods published in other journals. It is compiled by Norman S. Radin and H. A. Newman. (For Nos. 1–67 see JOURNAL OF LIPID RESEARCH, October, 1959.)**

68. ZUR THERMODYNAMISCHEN CHARAKTERISIERUNG DER SELEKTIVITÄT IN DER GASCHROMATOGRAPHIE. Bayer, E., and H. Röck. *Angew. Chem.* **71**: 407, 1959.

69. GAS CHROMATOGRAPHY. EFFECT OF SAMPLE SIZE ON HEIGHT OF EQUIVALENT THEORETICAL PLATE AND RETENTION VOLUME. Bethea, R. M., and M. Smutz. *Anal. Chem.* **31**: 1211, 1959.

70. DESIGN CONSIDERATIONS OF GAS CHROMATOGRAPHY SYSTEM EMPLOYING HIGH EFFICIENCY GOLAY COLUMNS. Condon, R. D. *Anal. Chem.* **31**: 1717, 1959.

71. GAS CHROMATOGRAPHIC EVIDENCE FOR INTRAMOLECULAR HYDROGEN BONDING WITH DOUBLE BONDS. DePuy, C. H., and P. R. Story. *Tetrahedron*, 20 (1959).

72. GAS-LIQUID CHROMATOGRAPHY OF NATURAL PRODUCTS AND THEIR DERIVATIVES. Eglinton, G., R. J. Hamilton, R. Hodges, and R. A. Raphael. *Chem. & Ind. London*, 955 (1959).

73. POLYESTER LIQUID PHASES IN GAS-LIQUID CHROMATOGRAPHY. Horning, E. C., E. A. Moscatelli and C. C. Sweeley. *Chem. & Ind. London*, 751 (1959).

74. GAS AND LIQUID ELUTION CHROMATOGRAPHY. QUANTITATIVE DETECTOR EVALUATION. Johnson, H. W., Jr., and F. H. Stross. *Anal. Chem.* **31**: 1206, 1959.

75. GAS-LIQUID CHROMATOGRAPHY OF FATTY DERIVATIVES. II. ANALYSIS OF FATTY ALCOHOL MIXTURES BY GAS-LIQUID CHROMATOGRAPHY. Link, W. E., H. M. Hickman and R. A. Morrissette. *J. Am. Oil Chemists' Soc.* **36**: 300, 1959.

76. THE COMPARISON OF DETECTORS FOR GAS CHROMATOGRAPHY. McWilliam, I. G. *J. Appl. Chem. London* **9**: 379, 1959.

77. NYLON CAPILLARY COLUMNS FOR USE IN GAS-LIQUID CHROMATOGRAPHY. Scott, R. P. W. *Nature* **183**: 1753, 1959.

78. ANALYSIS BY GAS CHROMATOGRAPHY OF A 'PURE' SAMPLE WITH AN 'IMPURE' CARRIER. Willis, V. *Nature* **183**: 1753, 1959.

79. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Bligh, E. G., and W. J. Dyer. *Can. J. Biochem. Physiol.* **37**: 911, 1959.

80. PHOTOMETRIC MILK FAT DETERMINATION. Haugaard, G., and J. D. Pettinati. *J. Dairy Sci.* **42**: 1255, 1959.

81. A UNIFORM BASIS FOR REPORTING ANALYTICAL DATA ON FATTY MATERIALS. Showell, J. S. *J. Am. Oil Chemists' Soc.* **36**: 343, 1959.

82. A SIMPLE TECHNIC FOR STAINING FAT WITH OIL-SOLUBLE AZO DYES. Sills, B., and W. H. Marsh. *Lab. Invest.* **8**: 1006, 1959.

83. SEPARATION AND CHEMICAL ASSAY OF LIPIDE CLASSES. E. Van Handel. *J. Am. Oil Chemists' Soc.* **36**: 294, 1959.

84. UNE MÉTHODE POUR L'ÉTUDE DES LIPIDES HEMATIQUES —I. Vergine, A. *Biochem. Pharmacol.* **2**: 68, 1959.

85. DESATURATION OF STAINING SOLUTIONS FOR LIPIDS: A MEANS OF AVOIDING PRECIPITATION ON STAINED SECTIONS. Vlachos, J. *Stain Technol.* **34**: 292, 1959.

86. ADSORPTIONSCHROMATOGRAPHISCHE UNTERSUCHUNG VON SERUMLIPIDEN AUF KIESELGELPLATTEN. Weicker, H. *Klin. Wochschr.* **37**: 763, 1959.

87. USE OF PORTER-SILBER AND SCHIFF REAGENTS AS SPOT TESTS FOR STEROIDS APPLIED ON PAPER AND THEIR APPLICATION TO THE STUDY OF RAT ADRENAL LIPIDS. Birmingham, M. K. *Nature* **184**: 67, 1959.

88. ERRORS IN THE DETERMINATION OF SMALL AMOUNTS OF URINARY STEROIDS. Bulbrook, R. D., Y. A. Leon and B. S. Thomas. *J. Endocrinol.* **18**: xxix, 1959.

89. Die Ir-Spektren von Steroiden mit Hydroxyl-, Ketal- oder Acetat-Gruppen. I. Hydroxyprogesterone. Heller, K. *Z. Naturforsch.* **14b**: 298, 1959.

90. Determination of Individual Adrenocortical Steroids in Urine of Pregnant Women. Johnson, D. F., D. François and E. Heftmann. *Acta Endocrinol.* **32**: 8, 1959.

91. An Improved, Rapid Method for Free and Conjugated 17-Hydroxycorticosteroids in Plasma. Kornel, L. *J. Lab. Clin. Med.* **54**: 659, 1959.

92. An Improved, Rapid Method for Free and Conjugated 17-Hydroxycorticosteroids in Urine. Kornel, L. *Metabolism, Clin. and Exptl.* **8**: 432, 1959.

93. Rapid Separation and Quantitation of Three Phenolic Estrogens by Paper Chromatography. Porter, C. W., E. L. Clark and G. E. Block. *J. Lab. Clin. Med.* **54**: 471, 1959.

94. Methoden zur Corticoidbestimmung im Menschlichen Harn. Überblick und Vergleichende Untersuchungen. Raftopoulo, R., H. J. Staudinger and L. Weissbecker. *Clin. Chim. Acta* **4**: 463, 1959.

95. A Simplified Method for the Simultaneous Determination of 17-Ketosteroids, Dehydroepiandrosterone, and 17-Hydroxycorticosteroids in Serum. Saier, E. L., E. Campbell, H. S. Strickler, and R. C. Grauer. *J. Clin. Endocrinol. and Metabolism* **19**: 1162, 1959.

96. Chromatography of Urinary Ketosteroids. Sheath, J. B. *Australian J. Exptl. Biol. Med. Sci.* **37**: 147, 1959.

97. Factors in the Colorimetric Estimation of 17-Ketosteroids in Urine. Sheath, J. B. *Australian J. Exptl. Biol. Med. Sci.* **37**: 133, 1959.

98. 16 α-Hydroxy Steroids II. Partition Chromatography of Triamcinolone and Related Steroids. Smith, L. L., T. Foell, R. De Maio, and M. Halwer. *J. Am. Pharm. Assoc. Sci. Ed.* **48**: 528, 1959.

99. Chemical Determination of Aldosterone in Urine. Sobel, C., R. J. Henry, O. J. Golub, and M. Rudy. *J. Clin. Endocrinol. and Metabolism* **19**: 1302, 1959.

100. Charakterisierung Einfach Ungesättigter Steroide durch Ihr Absorptionsspektrum im Kurzwelligen Ultraviolett. Stich, K., G. Rotzler and T. Reichstein. *Helv. Chim. Acta* **42**: 1480, 1959.

101. The Determination of Bile Acids in Bile and Duodenal Contents by Quantitative Paper Chromatography. Bile Acids and Steroids 71. Sjövall, J. *Clin. Chim. Acta* **4**: 652, 1959.

102. Differential Microdetermination of Bile Acids in Bile by Paper Chromatography. Watanabe, N. *J. Biochem. Tokyo* **46**: 681, 1959.

103. A Comparison of the Methods of Bloor and Schoenheimer-Sperry in the Estimation of Cholesterol in Serum. Duffie, M. J., and J. L. Guravich. *Am. J. Clin. Pathol.* **32**: 92, 1959.

104. Le Dosage du Cholestérol Sanguin par Colorimétrie sur le Digitonide. Améliorations Opératoires, en Particulier dans les Cas d'Hyperlipémies Élevées. Herbain, M. *Bull. soc. chim. biol.* **41**: 821, 1959.

105. The Use of Chromatography Paper in the Study of Sterol Adduct Formation. Meijer, J. W. A., and C. J. F. Böttcher. *Rec. trav. chim.* **78**: 622, 1959.

106. Estimation of Serum Cholesterol. Morris, T. G. *J. Physiol. London* **147**: 41P, 1959.

107. Structures and Infrared Spectra of Monomethyloctadecanoic Acids. Abrahamsson, S., and I. Fischmeister. *Arkiv Kemi* **14**: 57, 1959.

108. Identifizierung Organischer Verbindungen XXV. Identifizierung und Trennung Aliphatischer Alkohole C_1-C_{18} Mittels Papierchromatographie. Borecky, J., J. Gasparic and M. Vecera. *Collection Czechoslov. Chem. Communs.* **24**: 1822, 1959.

109. Paper Chromatography of Saturated Fatty Acids. Buchanan, M. A. *Anal. Chem.* **31**: 1616, 1959.

110. Separation of Fatty Acids from Tubercle Bacillus by Gas Chromatography: Identification of Oleic Acid. Cason, J., and P. Tavs. *J. Biol. Chem.* **234**: 1401, 1959.

111. Microdetermination of Unsaturation in Organic Compounds. Chaphekar, M. R., and T. S. Gore. *Mikrochim. Acta*, 664 (1959).

112. Studies on the Metal Complexes of Hydroxamic Acids. Coloured Complexes of Iron, Vanadium and Molybdenum with *Iso*nicotinohydroxamic Acid and Their Analytical Uses. Dutta, R. L. *J. Indian Chem. Soc.* **36**: 285, 1959.

113. Bestimmung von Nichtesterifizierten Fettsäuren im Blut und in Geweben. Dzúrik, R., P. Kovács and T. R. Niederland. *Naturwissenschaften* **46**: 327, 1959.

114. Reaktion Ungesättigter Fettsäuren mit Quecksilber(II)-Acetat; Anwendung für Präparative Trennungen, I. Ernst, J., and A. Holger. *Chem. Ber.* **92**: 1427, 1959.

115. The Mass Spectra of Methyl Oleate, Methyl Linoleate, and Methyl Linolenate. Hallgren, B., R. Ryhage and E. Stenhagen. *Acta Chem. Scand.* **13**: 845, 1959.

116. Spectrophotometric Determination of Small Quantities of Esterified Fatty Acids in Human Plasma. Ivaldi, G., and I. Macrì. *Ital. J. Biochem.* **8**: 35, 1959.

117. Determination of the Degree of Unsaturation of Long Chain Fatty Acids by Gas-Liquid Chromatography. James, A. T. *J. Chromatog.* **2**: 552, 1959.

118. Determination of Small Amounts of Secondary Amine in High Molecular Weight Fatty Primary Amines. Milun, A. J., and J. P. Nelson. *Anal. Chem.* **31**: 1655, 1959.

119. A Modified Hydroxamic Acid Method for Determining Total Esterified Fatty Acids in Plasma. Morgan, D. M., and K. J. Kingsbury. *Analyst* **84**: 409, 1959.

120. Paper Chromatography of Volatile Fatty Acids. Mukerjee, H. *Anal. Chem.* **31**: 1284, 1959.

121. Glass Paper Chromatography of Long-Chain Fatty Acids, Brominated Derivatives, and Methyl Esters. Ory, R. L., W. G. Bickford and J. W. Dieckert. *Anal. Chem.* **31**: 1447, 1959.

122. Identifizierung der Fettsäuren C_8-C_{11} Mittels Papierchromatographie. Romaňuk, M. *Collection Czechoslov. Chem. Communs.* **24**: 2064, 1959.

123. Mass Spectrometric Studies. I. Methyl Esters of Saturated Normal Chain Carboxylic Acids. Ryhage, R., and E. Stenhagen. *Arkiv Kemi* **13**: 523, 1959.

124. Weitere Untersuchungen zum Nachweis Konjugiert-Ungesättigter Fettsäuren in den Menschlichen Serumlipiden. Schauenstein, E., and H. Buchta. *Klin. Wochschr.* **37**: 705, 1959.

125. Eine Neue Farbreaktion zum Nachweis Organischer Säuren auf Papierchromatogrammen. Schlögl, K. *Naturwissenschaften* **46**: 447, 1959.

126. A Simplified Spectrophotometric Determination of Ester Groups in Lipids. Snyder, F., and N. Stephens. *Biochim. et Biophys. Acta* **34**: 244, 1959.

127. Automatic Coulometric Micro-Determination of Unsaturation with Kinetic Proof of Real Equivalence-Point. Walisch, W., and M. R. F. Ashworth. *Mikrochim. Acta*, 497 (1959).

128. Zur Bestimmung der Veresterten Fettsäuren im Blutserum Unter Besonderer Berücksichtigung der Hydroxamsäuremethode. Weller, H. *Klin. Wochschr.* **37**: 951, 1959.

129. A Microcolorimetric Method for the Determination of Sorbitol, Mannitol, and Glycerol in Biologic Fluids. Bailey, J. M. *J. Lab. Clin. Med.* **54**: 158, 1959.

130. Determination of Glycerol in Blood-Glycerol Mixtures. Crutchfield, C. A., Jr., and H. A. Sloviter. *J. Lab. Clin. Med.* **54**: 478, 1959.

131. Chromatographic Analysis of Glycerides. Part I. Paper Chromatography of Synthetic and Natural Glycerides as Their Mercury Addition Compounds. Inoue, Y., and M. Noda. *Nippon Nôgei-kagaku Kaishi* **33**: 452, 1959.

132. The Separation of Glycerides by Crystallization in a Thermal Gradient. Magnusson, J. R., and E. G. Hammond. *J. Am. Oil Chemists' Soc.* **36**: 339, 1959.

133. Glyceride Structure of Vegetable Oils by Countercurrent Distribution. IV. Cocoa Butter. Scholfield, C. R., and H. J. Dutton. *J. Am. Oil Chemists' Soc.* **36**: 325, 1959.

134. Papierchromatographische Analyse von Plasmalogen. Hack, M. H., and V. J. Ferrans. *Z. physiol. Chem., Hoppe-Seyler's* **315**: 157, 1959.

135. A Micromethod for the Polarographic Determination of Serine. Ladik, J., and I. Szekacs. *Nature* **184**: 188, 1959.

136. Analysis of Milk Phospholipids by Chromatography and Infrared Spectrophotometry. Smith, L. M., and N. K. Freeman. *J. Dairy Sci.* **42**: 1450, 1959.

137. Anwendung der Gaschromatographie zur Identifizierung Einiger Terpene aus Ätherischen Ölen von Nadelbäumen *(Coniferae)*. Cvrkal, H., and J. Janák. *Collection Czechoslov. Chem. Communs.* **24**: 1967, 1959.

138. The Determination of Tocopherols in Oils, Foods and Feeding Stuffs. Analytical Methods Committee. *Analyst* **84**: 356, 1959.

139. Analysis of Fat Soluble Vitamins IV. Discussion of the Chemical Routine Determination of Vitamin D. Hanewald, K. H. *Rec. trav. chim.* **78**: 604, 1959.

140. Quantitative Determination of Carotene by Paper Chromatography. Jensen, A. *Acta Chem. Scand.* **13**: 1259, 1959.

141. Determination of Vitamin D in Multivitamin Mixtures after Separation by Partition Chromatography. Theivagt, J. G., and D. J. Campbell. *Anal. Chem.* **31**: 1375, 1959.

142. The Determination of Serum α- and β- Lipoprotein Cholesterol. Crawford, N. *Clin. Chim. Acta* **4**: 494, 1959.

143. Ultracentrifugal Method for Determination of Serum Lipoproteins. Del Gatto, L., F. T. Lindgren and A. V. Nichols. *Anal. Chem.* **31**: 1397, 1959.

144. The Determination of Egg in Certain Foods by Enzymic Hydrolysis of the Phospholipids. Casson, C. B., and F. J. Griffin. *Analyst* **84**: 281, 1959.

145. New Determination of Lecithinase A Activity. Wakui, K., and S. Kawachi. *Yakugaku Zasshi* **79**: 867, 1959.

146. Nephelometric Detection of Lipides in Chromatographic Column Effluents. Arcus, A. C. *Anal. Chem.* **31**: 1618, 1959.

147. Analysis of Plant Waxes by Means of Chromatography and X-Ray Diffraction. N. Wiedenhof. *J. Am. Oil Chemists' Soc.* **36**: 297, 1959.

Notice of Meeting

The Fifth Meeting of the International Conference on Biochemical Problems of Lipids will take place in Marseilles, France, July 21-23, 1960. Subject: "The Enzymes of Lipid Metabolism." Sections: (1) Techniques, (2) Hydrolysis, (3) Oxidative Degradation, (4) Biosynthesis. For information write Prof. P. Desnuelle, Faculté des Sciences, Place Victor Hugo, Marseilles, France.

Author's Correction

In the article entitled "Scintillation Counter for the Measurement of Radioactivity of Vapors in Conjunction with Gas-Liquid Chromatography," by G. Popják *et al.*, in the October, 1959, issue, footnote 1 (p. 34) gives 1172 as the type number of the ratemeter specified. Please note that the correct type number is N. 522.

INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4:** 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg.
gram	g.
milligram	mg.
microgram	μg.
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	$cm.^2$
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc.
microcurie	μc.
degrees of temperature	°C or °F

The expression mg.% should not be used, but should be written as mg. per 100 ml. For chemical nomenclature the JOURNAL will follow the conventions of *Chemical Abstracts*. The superscript ® should follow all trade names. When necessary for identification of other specific materials, use a footnote, including name and location of manufacturer.

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FOR JOURNAL ARTICLES: Author's last name, initials. *Name of journal* (abbreviated as in "List of Periodicals Abstracted," *Chemical Abstracts*, Supplementary Issue to Volume 50, 1956) volume number: page, year.
Example: Garn, S. M., A. B. Lewis and D. L. Polacheck. *Science* **128:** 1510, 1958.

FOR BOOKS: Author's last name, initials. *Title of Book.* City, Publishing House, year, page number.
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millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m.
centimeter	cm.
millimicron	mμ.
square centimeter	$cm.^2$
liter	l.
milliliter	ml.
cubic centimeter	cc. or $cm.^3$
microliter	μl.
counts per minute	cpm.
millicurie	mc
microcurie	μc.
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